Product Specificity of Rice Germ Lipoxygenase

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ABSTRACT

Incubation of linoleic acid with partially purified lipoxygenase from rice germ yielded a ratio of 9- to 13-hydroperoxides of linoleic acid of 97:3 as measured by high performance liquid chromatography. Under similar conditions, hematin gave the 9- to 13-hydroperoxides at a ratio of 51:49, and soybean lipoxygenase-a at 9:91. Infrared spectral analysis revealed *cis-trans* configuration to predominate in the reaction products with the rice germ enzyme as was with the soybean enzyme.

INTRODUCTION

Much work has been conducted on the positional and stereochemical features of the reaction catalyzed by lipoxygenases (EC 1.13.11.12) from various sources, since the positional and stereochemical specificity in the products of soybean lipoxygenase were first established by Hamberg and Samuelsson (1). The ratio of the two positional isomers formed from linoleic acid, 9- to 13-hydroperoxides, varies to no negligible extent with pH, oxygen concentration, temperature and other conditions of the incubation (2,3), but much more crucially with sources and homogeniety of the enzyme used (4-11). Lipoxygenase can be classified into three groups according to their product specificity observed when linoleic acid is used as the substrate: enzyme selectively producing the 9-isomer, enzyme selectively producing the 13-isomer, and enzyme forming both isomers. The hydroperoxide isomers are formed in a range of tissues and converted into their several secondary products. Among them are included those volatile carbonyl compounds we have detected in rice bran and cooked rice (12,13). The purpose of this investigation was to identify which hydroperoxide isomer is the predominant product with rice germ lipoxygenase compared to soybean lipoxygenase-a and hematin. According to our original designation (14), soybean lipoxygenase used in this study is referred to as lipoxygenase-a, which is identical with lipoxygenase-1 of Christopher et al. (15). The term isoenzyme is avoided since we still lack genetic data indicating the structural difference among the multiple forms of lipoxygenases (16).

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MATERIALS AND METHODS

Enzyme Purification

Soybean lipoxygenase-a was prepared by the method described previously (970 mkat/kg protein; one katal (symbol:kat) corresponds to the amount of activity that converts one mol of substrate (linoleate) per second) (14,17). Rice germ lipoxygenase was partially purified from germs of *Japonica* variety rice grains (Nipponbare) by ammonium sulfate fractionation, heat treatment, chromatography on DEAE-Sephadex A-50 column, and gel filtration on Sephadex G-100 column. The enzyme preparation had a specific activity of 140 mkat/kg protein. Enzymatic characteristics of the purified enzyme will be detailed elsewhere.

Reaction Mixture

Fifty μmol sodium linoleate were incubated with rice germ lipoxygenase (100 nkat) in 10 ml potassium phosphate buffer (0.1 M, pH 7.0) or with soybean lipoxygenase-a (20 nkat) in 10 ml sodium borate buffer (0.1 M, pH 9.0). Five μmol sodium linoleate were incubated with 114 μmol hematin in 200 ml sodium borate buffer (0.1 M, pH 9.0). The reactions were run for stated times at an ambient temperature (22 ± 3C) with a steady stream of oxygen, and terminated by acidifying the incubation mixtures to pH 3 with HCl. The reaction products and unconverted substrate were extracted with chloroform/methanol (2:1). The volume of the extracts was reduced with a stream of nitrogen. The product mixtures thus prepared were stored below -20 C as diluted solutions in n-hexane (2-10 mg linoleate hydroperoxides/ml).

High Performance Liquid Chromatography (HPLC)

HPLC was carried out at an ambient temperature (22 ± 3C) on a column of Zorbax SIL (2.1 mmϕ x 150 mm) in Shimadzu-Dupon High Performance Liquid Chromatograph LC-1.

Eluant (n-hexane/1% absolute ethanol) was delivered at 1 ml/min, and the effluent was monitored at 234 nm. The area under the individual peak was determined by an integrator (Shimadzu Chromatopac-EIA). To obtain workable quantities of individual components, ca. 1 mg of the product mixture was repeatedly injected and the components were collected separately.

Gas Chromatography-Mass Spectrometry (GC-MS)

The hydroperoxides in the product mixtures were reduced by $NaBH_4$ in methanol. The resulting hydroxy fatty acids were recovered by acidifying and extracting with ethyl ether and were converted into their methyl esters with diazomethane. The methyl esters dissolved in methanol were hydrogenated with a platinum catalyst. The hydrogenated compounds were trimethylsilylated with TMS-BA kit (Tokyo Kasei Kogyo Co., Ltd.) just before the GC-MS analysis. GLC separation was carried out using He gas as carrier. The column temperature was programmed from 220 to 260 C at 3C per min. The gas chromatograph, Shimadzu GLC-9000, was coupled to a mass spectrometer which was interfaced to a computer system (Peak matcher MID-PM, GC-MS Pack-300DG).

Infrared (IR) Spectroscopy

IR spectra were recorded on a Hitachi IR Spectrometer with micro analytical units. The product mixtures were analyzed as liquid films on sodium chloride plates.

RESULTS

Formation of Linoleate Hydroperoxides

The oxygenation reaction of linoleate was followed by monitoring the absorbance at 234 nm (Fig. 1). Based on a molar absorbance of 25,000 $mol^{-1}cm^{-1}$ for the conjugated diene hydroperoxides (18), a maximal yield of 90% was attained with rice germ lipoxygenase for a 60 min incubation, and an 80% yield with soybean lipoxygenase-a for a 15 min incubation, while a 60% conversion was effected with hematin after 150 min incubation. Since absorbance decreased after these times of incubation, these were optimal reaction times for the respective systems.

IR Spectroscopy of Unresolved Reaction Products

IR spectral analysis has been successfully employed for designating the geometric configuration of the isolated isomers of the linoleate hydroperoxides, and also for approximating the isomeric ratio in the mixtures (19-21). The reaction product mixtures with soybean lipoxygenase-a and rice germ lipoxygenase represented two absorption bands at 943 and 977 cm^{-1} which are characteristic of (Z,E)-conjugated diene configuration. Use of (Z) and (E) denotes *cis* and *trans*, respectively, as set forth by the IUPAC (22). The products obtained under the hematin catalysis had a distinct absorption band at 985 cm^{-1}, accompanied by two blurred bands around 943 and 977 cm^{-1}, the spectrum suggesting both (E,E) and (Z,E) configurations to be present (19).

Separation of Hydroperoxide Isomers by HPLC

In Figure 2 are shown typical HPLC separations of the hydroperoxides obtained from incubating linoleate with rice germ lipoxygenase, soybean lipoxygenase-a, and hematin. By referring to the results of previous investigators (4,5), we assigned the three major peaks appearing between 20 and 35 min of elution to 13-hydroperoxy-9(Z),11(E)-octadecadienoic acid, 13-hydroperoxy-9(E),11(E)-octadecadienoic acid, and a mixture of 9-hydroperoxy-10(E),12(E) and (Z)-octadecadienoic acids, respectively. These assignments were consistent with the GC-MS analysis of the respective peak components obtained by repeated HPLC on a preparative scale.

The distribution of the hydroperoxide isomers was determined by measuring the peak areas in the chromatograms shown in Figure 2, and the figures thus obtained are listed in Table I. Rice germ lipoxygenase at pH 7.0 formed almost exclusively the 9-hydroperoxide in contrast to soybean enzyme, which catalyzed at pH 9.0 the conversion of linoleate into

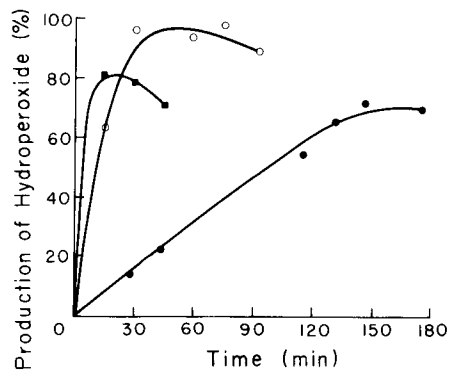

FIG. 1. Linoleate hydroperoxide production during incubation of linoleate with rice germ lipoxygenase (○), soybean lipoxygenase-a (■) and hematin (●). Incubation conditions are described under "MATERIALS AND METHODS."

mainly 13-hydroperoxides. The ratio of 13- to 9-hydroperoxides produced by the soybean enzyme is in substantial agreement with published data (6,7). Hematin yielded an equal mixture of 9- and 13-hydroperoxides at equal proportions of (E,Z) and (E,E) components.

Positional Specificity Determined by GC-MS

HPLC analysis revealed that the 9-hydroperoxide is the predominant product in the rice germ lipoxygenase-linoleate reaction. This positional isomerism was further identified using GC-MS. GC separated the derivatives of the reaction mixtures into the components corresponding to the unreacted substrate, the reaction products and unidentified minor products. The second peak, which could be hardly separated any further by GC procedure, proved on MS analysis to contain both the 9- and 13-isomers. The peak material yielded fragment ions diagnostic of methyl 9-trimethylsilyloxystearate at m/e 229 and 259, and those diagnostic of methyl 13-trimethylsilyloxystearate at 173 and 315. The mass peaks arising from the 13-isomer were discerned in a late eluting fraction, partially resolved from those peaks from the predominant 9-isomer (Fig. 3). With the assumption that both isomers show identical probabilities of fragmentation (23), the mass fragmentography allowed us to estimate the ratio of 9- to 13-isomers to be 88:12, the value being different from that estimated by HPLC (Table I). The detected difference conceivably indicates that some isomerization of the hydroperoxides could have occurred during the derivatization procedures to affect the determination of the positional specificities.

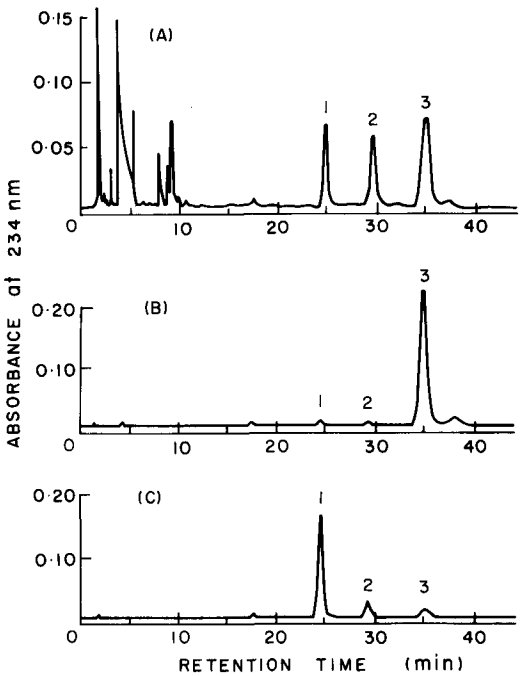

FIG. 2. High performance liquid chromatography of linoleate hydroperoxide isomers produced by incubation of linoleate with hematin (A), rice germ lipoxygenase (B) and soybean lipoxygenase-a (C). Peak 1, 13-(Z,E)-hydroperoxide: peak 2, 13-(E,E)-hydroperoxide: and peak 3, a mixture of 9-(E,Z)- and 9-(E,E)-hydroperoxides.

DISCUSSION

In Figure 1, prolonged reaction times resulted in decreasing absorbance at 234 nm. The phenomenon is indicative of hydroperoxide decomposition. As enzymes catalyzing the isomerization and decomposition of the hydroperoxides have been reported to occur widely in plants, their possible contamination in the lipoxygenase preparations may probably be responsible for the observed absorbance decrease. Apart from these contaminants, however, lipoxygenase itself can catalyze a homolytic degradation of the products of its own reactions (24,25). Therefore, the absorbance decrease may also be attributable to a lipoxygenase-catalyzed degradation of the hydroperoxides, which takes place concurrently with the enzymatic oxygenation reaction under the conditions used in this study.

TABLE I

Percentage of 9- and 13-Hydroperoxide Isomers Derived from Linoleate[a]

	13-(Z,E)	13-(E,E)	9-(Z,E) & 9-(E,E)
Rice germ lipoxygenase	2	1	97
Soybean lipoxygenase-a	80	11	9
Hematin	24	25	51

[a]Calculated from the chromatograms given in Figure 2.

The HPLC procedure used in this study, a modified procedure of Chan and Prescott (4) (without methylation of the samples), could resolve the 13-isomers into (E,Z) and (E,E) geometric components but not the 9-isomers, while the 9-isomers were separated from the resolved pair of the 13-isomers. Evidence is accumulating which suggests that lipoxygenase-linoleate reaction produces exclusively (Z,E) hydroperoxides, and (E,E) components are mostly the isomerization products of the (Z,E) components. This current concept leads to the hypothesis that the predominant geometric isomer of the 9-hydroperoxides formed by the rice germ lipoxygenase is of (E,Z) configuration, if not entirely, as inferred by Pattee and Singleton (5). Support is lent for this hypothesis by its IR spectrum, which is very similar to that of the products obtained with soybean enzyme in lacking a characteristic absorption band at 985 cm^{-1} for (E,E) configuration. The hypothesis is strengthened by the collateral evidence that the rice germ enzyme activity is optimal at neutral pH like those enzymes specific for (Z,E) 9-hydroperoxide formation, such as corn germ (2), potato tuber (9), barley (10) or tomato fruit (11).

Positional specificity of the rice germ enzyme seems to lack a general dependence on pH of the incubation mixture. HPLC analysis revealed that at pH 9.0 the enzyme reaction, while proceeding at a rate of only 4% as much as observed at pH 7.0, yielded the hydroperoxides at a positional isomer ratio similar to that established at pH 7.0. Contrary to this finding are the results of Veldink et al. (2), and Singleton et al. (8) that both corn germ and peanut lipoxygenases show a significant pH dependence in product specificity of linoleate oxygenation. However, these observations are more likely to reflect a composite pH-activity profile of two or more enzymes with different pH dependence and different positional specificity.

A uniform distribution of the hydroperoxide isomers was observed in the present study for the hematin oxidation of linoleate, implying oxygenation to occur equally at position 9 and 13 so as to form equal proportions of the positional isomers and, for both positional isomers, equal distributions of geometric isomers. The result we obtained varies from the finding of Chan et al. (26) that autoxidation as well as catalytic oxygenation of linoleate with hemo-proteins and Cu^{2+} and Fe^{2+} ions yield possibly the 13-hydroperoxide, a regio-selectivity that resembles that of lipoxygenase of a-type soybean enzyme. Assuming that hematin accelerates the isomerization of the hydroperoxide products, then the isomeric distribution we observed might simply reflect the equilibration attained under those conditions rather than random reaction of molecular oxygen with linoleate. This assumption agrees with the evidence of Chan et al. (27) for thermal isomerization of the hydroperoxides.

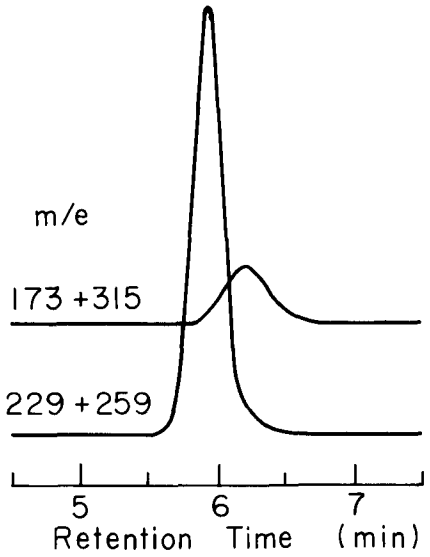

FIG. 3. Mass fragmentography of methyl trimethylsilyloxystearates derivatized from linoleate hydroperoxides produced by incubation of linoleate with rice germ lipoxygenase.

The 9-hydroperoxide is possibly not a primary direct precursor for hexanal, which is found as one of the characteristic components accumulating on storage of rice grain. It has to be established whether hexanal production in rice grain is due to a sequential enzyme system starting with lipoxygenase-oxidation of linoleate at carbon position 9 and ending with isomerization to 13-hydroperoxide and decomposition by a specific cleavage system. The evidence is yet lacking for isomerization of 9- to 13-hydroperoxide in rice germ.

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Interaction of Porcine Pancreatic Colipase with a Nonionic Detergent, Triton X-100: Spectrophotometric Studies

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ABSTRACT

Strong perturbation of the ultraviolet spectrum of the tyrosines of porcine pancreatic colipase A is observed in the presence of Triton X-100 at concentration above the critical micellar concentration. Spectrophotometric titration of the phenolic groups of the protein shows that the apparent pKa value for two tyrosines is about 10.3, while the third tyrosine has a higher pKa value above 11.6. This residue is still protonated at pH 13 in the presence of Triton X-100. All perturbations induced by the nonionic detergent can be interpreted as resulting from interactions between colipase and Triton X-100 molecules at a hydrophobic site of the protein that includes the tyrosine residues. Results obtained in studies with Triton X-100 are similar to those already reported by Sari et al. (Eur. J. Biochem. 58:561 (1975)) on the interaction of colipase with taurodeoxycholate. It is likely that the binding of both types of detergent occurs at the same specific site on the protein molecule. Data presented in this communication give further support to the hypothesis that a hydrophobic domain (residues 49-57), including all three tyrosines of the colipase molecule, participate to the well characterized interaction of the lipase cofactor with triglycerides at lipid-water interfaces.

INTRODUCTION

Colipase is a small protein acting as a cofactor of pancreatic lipase (1-3). Pure porcine and equine colipases have been recently isolated from pancreatic tissue homogenized in the presence of a nonionic detergent, Triton X-100 (4,5). The conditions used for the extraction of colipase were adapted from the detergent methods previously described for the separation of lipid interacting proteins. It was found that addition of the detergent not only resulted in a marked increase in the amount of extractable colipase but also in the protection of the protein against proteolytic cleavage in the amino-terminal region of the polypeptide chain; in particular, at the Arg_5-Gly_6 bond. The protective effect of the nonionic detergent was interpreted as resulting from molecules or aggregates of Triton X-100 interacting with the colipase molecule, although there was no evidence for such interaction.

It is well documented from the reports by the groups of Desnuelle (6-8) and Borgstrom (9-11) that colipase forms stable associations with conjugated bile salts (one molecule of colipase per micelle of taurodeoxycholate). Spectrophotometric studies of the colipase-taurodeoxycholate system allowed Sari et al. (8) to present the first experimental evidence showing that one or several of the tyrosine residues of colipase participate in the protein-detergent interaction. More recently, studies carried out on the same system by proton nuclear magnetic resonance spectroscopy have confirmed and extended the results concerning the participation of aromatic residues in the interaction of colipase with micelles of taurodeoxycholate (12,13). It has been shown that these residues are part of a hydrophobic site of

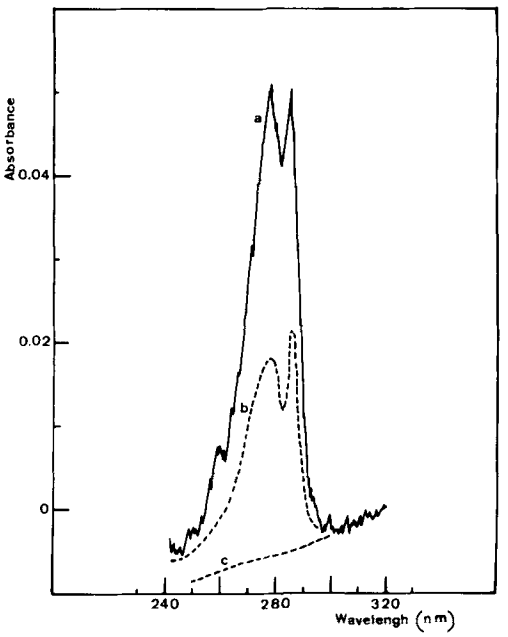

FIG. 1. Triton X-100 perturbation difference spectrum of porcine colipase A. (a) colipase (0.22 mM); (b) tyrosine (0.20 mM); and (c) ribonuclease (0.20 mM). Spectra were recorded at room temperature (22° ± 1°) within 10 min of mixing colipase in 50 mM Tris HCl pH 8.1 containing 100 mM NaCl and Triton X-100 at the final concentration of 0.5 mM.

the cofactor molecule interacting with the anionic detergent in the form of micelles (14).

Little information is presently available on the interaction of colipase with nonionic detergents. It was established that Triton X-100 and Brij 35 are strong inhibitors of the hydrolysis of water insoluble triglycerides by pancreatic lipase (15,16). Complete inhibition occurred at concentration well below the critical micellar concentration. This inhibition could be reversed by addition of colipase only in a narrow concentration range of Triton X-100, in contrast to the anionic taurodeoxycholate. Interpretation of this behavior remained difficult since no direct evidence of any interaction of colipase with nonionic detergents could be obtained in gel filtration experiments (16,17).

Results presented in this communication show that colipase interacts with Triton X-100. Protein-detergent interactions are associated with perturbation of the aromatic side-chains of the tyrosines of colipase. Comparison with data previously obtained in studies with the colipase-taurodeoxycholate system suggests that one specific domain of the colipase molecule interacts with detergents and participates in the formation of mixed micellar aggregates.

MATERIALS AND METHODS

Proteins and Chemicals

All experiments were performed with porcine colipase A prepared as previously described (4). Protein concentration was estimated spectrophotometrically at 280 nm using an extinction coefficient ($A_1^{1\%}{}_{cm}$) of 3.6, or by the colorimetric method of Lowry. Triton X-100 (p-(1,1,3,3-tetramethylbutyl)-phenoxypolyoxyethylene glycol) with an average of 8.5 oxyethylene units per mole (molecular weight: 624 dalton), was obtained from Fluka. The apparent critical micellar concentration (CMC) of Triton X-100 in water (0.130 mg per ml or 0.2 mM) was determined by the spectral method in aqueous solutions of iodine (18). The concentration of aqueous solutions of Triton X-100 was estimated spectrophotometrically at 274 nm using an extinction coefficient ($A_1^{1\ mg/ml}{}_{cm}$) of 2.32 (19).

Difference Spectral Measurements

The procedure used to measure the difference spectra was essentially that described by Herskovits (20). Ultraviolet difference spectra were obtained on a Cary 14 recording spectrophotometer having a 0-0.1 expanded absorbance range. Tandem cells of 1.0 cm path length (0.5 cm per compartment) were used. The spectra were recorded at room temperature ($21^\circ \pm 1^\circ C$). Stock solutions of porcine colipase (0.25 mM) and Triton X -100 (1% or 16 mM) were both prepared in 50 mM Tris HCl buffer pH 8.1 containing 100 mM NaCl. The final solutions were made by mixing 1 ml of the protein solution with volumes of detergent up to 100 μl. Difference spectra were recorded 10 min after the addition of Triton X-100. All values were corrected for dilution.

Titration of tyrosine

Tyrosines were titrated as described by Tanford (21). Solutions of colipase (0.10 mM in 0.25 M KCl) were prepared in the absence and in the presence of 1.5 mM Triton X-100. The ionic strength of the sample was maintained at 0.25, while the pH varied from 8 to 13, by adding 0.1 M NaOH in 0.15 M KCl. Difference absorbance of alkaline minus neutral solutions were determined at 295 nm 10 min after the addition of alkali. No significant increase in absorbance was observed after this period of time. The number of moles of tyrosine ionized per molecule of protein was calculated using an extinction coefficient of 2300 cm^{-1} M^{-1} for the phenoxide ion and an average value of 11,000 dalton for the molecular weight of colipase.

RESULTS AND DISCUSSION

Difference Spectra

The ultraviolet difference spectrum of porcine colipase in the presence of Triton X-100 is shown in Figure 1, curve a. For purpose of comparison, the difference spectrum of free tyrosine is also presented (curve b). The difference spectrum of porcine colipase shows two peaks in the 277-279 nm region and in the 285-287 nm region. These difference maxima are indicative of the changes that occurred in the environment of the tyrosine residues of the protein upon addition of Triton X-100 (20). The effect of the concentration of the nonionic detergent on the absorbance difference at 278 nm is shown in Figure 2. No perturbation of the tyrosines could be detected below the CMC of Triton X-100. The absorbance difference increased with increasing concentrations of detergent within the range 0.2 mM to 0.6 mM. Data presented in Figure 1 and Figure 2 are similar to those previously reported by Sari et al. (8) who studied the binding of colipase with micelles of taurodeoxycholate. It appears that colipase-Triton X-100 interactions bring the tyrosines of the coprotein in a more hydrophobic environment at proximity of the

apolar heads of the organized detergent molecules when mixed aggregates are being formed. From the primary structure established by Charles et al. (22), it can be observed that all three tyrosines of the colipase molecule (Tyr 53, Tyr 56 and Tyr 57) are found in a segment of nine residues with a large proportion of apolar side chains (Ala_{49}-Phe-Thr-Leu-Tyr-Gly-Val-Tyr-Tyr_{57}). It is reasonable to assume that the particular location of the tyrosines facilitates the interactions of the aromatic side chains with the hydrophobic moieties of detergent molecules in the process of comicellization. Furthermore, the finding that the tyrosines of colipase are similarly altered in micellar solutions of both anionic or neutral detergents, suggests that micellar aggregation occurs at the same site on colipase. Although no perturbation could be detected spectrophotometrically below the CMC, the possibility that individual molecules or aggregates of smaller size (premicelles) of Triton X-100 cannot be ruled out. Such binding could account for the failure of colipase to activate lipase in the presence of nonionic detergent even at concentration below the CMC (15). Notice can be made that bovine ribonuclease, a pancreatic protein of similar size with four tyrosines and no tryptophan, shows no perturbation of the aromatic chromophores when added with Triton X-100 (Fig. 1, curve c).

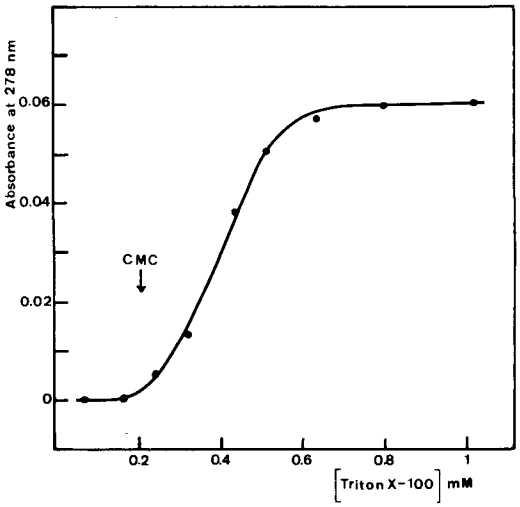

FIG. 2. Effect of the concentration of Triton X-100 on the absorbance difference at 278 nm of porcine colipase A. Concentration of colipase: 0.22 mM in 50 mM Tris-HCl pH 8.1 containing 100 mM NaCl.

Ionization of Tyrosines

The ionization curve of the phenolic groups of porcine colipase was determined in the absence and in the presence of 1.5 mM Triton X-100. Results are presented in Figure 3. In the absence of detergent (curve a), the data in the pH region 9 to 11.4 can be fitted with the theoretical curve of the dissociation of two phenolic groups with apparent pKa values of ca. 10.3 The apparent pKa value of the third tyrosine should be above pH 11.6. These observations are in good accordance with the recently reported results of the titration of the aromatic residues of porcine pancreatic colipase A by proton NMR spectroscopy (14,23). In these studies, it was established that two tyrosines with pKa values of 10.3 and 10.4 are readily exposed to the solvent while the third tyrosine titrates with an apparent pKa value of 11.3 indicative of a buried residue. In addition, titration profile of this latter group could not be fitted with a theoretical Henderson-Hasselbach equation. This likely reflects that conformational changes, induced by high pH, are required for the unmasking of this residue. A first evidence showing that partial unfolding of the colipase molecule occurs at high pH values is given by the observed time-dependence of the spectrophotometric titration of the third tyrosine above pH 11.3. When titration is performed in the presence of 1.5 mM Triton X-100, it appears that the two tyrosines with pKa values ca. 10.3 remained accessible to the solvent (Fig. 3, curve b). In contrast, in the same conditions, the masked phenolic group is still buried to hydroxyde ions after 10 min exposure at pH 11.3, which is indicative of a

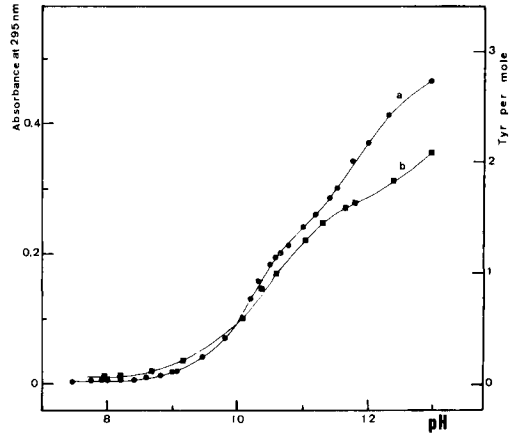

FIG. 3. Titration of the phenolic groups of porcine colipase A in the absence (a) and in the presence (b) of Triton X-100. Concentration of colipase: 0.075 mM in 0.25 M NaCl. Concentration of Triton X-100: 1.5 mM. Absorbance was measured 10 min after the addition of alkali.

stabilization of the native conformation of the colipase in the protein-detergent complex.

The results reported in this communication are in very good accordance with those previously obtained by Sari et al. (8) and those more recently presented by Sari et al. (24), who studied the accessibility of the tyrosine residues of colipase to small ions and neutral molecules by solvent perturbation and fluorescence quenching in the absence and in the presence of micelles of taurodeoxycholate. The results presented here support the conclusion that ionic and nonionic detergents bind to a hydrophobic site of the colipase molecule which includes the tyrosine residues of the protein. However, in contrast to taurodeoxycholate, one molecule of colipase does not form a stable association with one micelle of Triton X-100 as recently shown by Borgstrom et al. (17) in gel filtration experiments. It is likely that the interaction of colipase with detergents merely reflects the lipid affinity of this protein, and it can thus be assumed that the potential detergent binding site is part of a specific domain of the colipase molecule allowing colipase to adsorb at the triglyceride-water interface.

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We wish to express our thanks to Professor P.J. Cozzone for many helpful discussions and suggestions in the course of these studies.

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Lipofuscin in Vitamin E Deficiency and the Possible Role of Retinol

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ABSTRACT

This study was designed to determine if the vitamin A status of rats could affect the degree of lipofuscin formation in vitamin E deficient rats, inasmuch as an earlier report proposed a retinoyl complex in human brain lipofuscin pigment. Female rates were depleted of vitamin E from weaning while being maintained on different intakes of vitamin A (0, 0.8 and 8.0 mg/kg diet). The amount of lipofuscin present in the uterus was estimated at intervals between 2 and 8 months by visual observations, by histological fluorescence and by organic solvent extractable fluorescence. There was no difference in pigment deposition by any of the three criteria used, whether the animals were made retinol deficient and maintained on retinoic acid or were fed a low or high intake of retinol. Organic solvent extractable fluorescence was a poor indicator of the degree of pigment deposition in the uterus. It appears unlikely that retinol is a significant component of lipofuscin pigment in this tissue.

INTRODUCTION

Interest in the inert fluorescent pigment, termed lipofuscin or ceroid, that accumulates in animal tissues with age or at an accelerated rate in vitamin E deficiency, has recently been focused on its chemical and physical properties. The pigment is generally considered to be a product of lipid peroxidation involving in part a Schiff's base-type structure (1), but a recent report proposed that human brain pigment contained a moiety derived from vitamin A or retinol (2). If confirmed, this would add a new consideration to the origin and structure of the fluorescent pigment. In this report, we have investigated the possible involvement of vitamin A in the formation of the lipofuscin pigment which accumulates in the uterus of vitamin E deficient rats.

EXPERIMENTAL PROCEDURES

Animals and Diets

Weanling female rats of the Sprague Dawley strain were obtained from Taconic Farms (Germantown, NY) and placed in individual stainless steel suspended cages with food and water provided ad libitum. Temperature was controlled at 24 ± 1 C with a fixed 12 hr light-dark cycle. The basal diet was free of all forms of vitamin A and vitamin E and had the following composition in percent: vitamin-free casein, 20.0; mineral mixture with trace minerals (3), 3.6; vitamin mixture (3) with no A or E, 2.0; vitamin E-stripped corn oil with 0.02% BHT, 10.0; cellulose, 4.0; sucrose, 60.4. To this diet were added retinol as retinyl palmitate in the form of a stabilized powder, or retinoic acid in ethanol; control diets had vitamin E added as RRR, α-tocopheryl acetate. The amounts of the various supplements are indicated below. After 2, 5, and 8 months, the animals were sacrificed and tissues taken for histology or frozen at -20 C until analyzed.

Three experiments were carried out with vitamin E deficient rats comparing the effects of various levels of retinol or retinoic acid on lipofuscin formation: (1) a minimal intake of retinol, 0.8 mg/kg diet vs. a high intake, 8.0 mg/kg; (2) a minimal retinol level, 0.8 mg/kg vs. no retinol but with retinoic acid, 4 mg/kg, to maintain normal growth and (3) a moderate level of retinol, 2 mg/kg vs. no retinol but with retinoic acid, 4 mg/kg.

Histological Fluorescence

Tissue was fixed for 30 min at room temperature in a solution of 2.5% glutaraldehyde and 6% sucrose buffered to pH 7.2 with 50 mM sodium cacodylate, then fixed for another 30 min in 10% phosphate-buffered formalin. Frozen sections 10 μm thick were prepared and mounted on slides in glycerol. They were examined for autofluorescence under a Leitz Orthoplan microscope equipped with a mercury vapor lamp and a GB 38 heat filter. A 2 mm UGI excitation filter (peak transmission 360 nm) and a 460 nm barrier filter were used to localize and characterize the autofluorescence typical of lipofuscin.

Extraction of Tissue Fluorescence

Initially, the procedure of Csallany and Ayaz (4) was used to quantify the lipid soluble fluorescent material. In this procedure, a chloroform-methanol extract of tissue (0.3-0.5 g of uterus) was washed with relatively large volumes of water (50 ml five times) and the chloroform soluble material chromatographed

on a column of Sephadex LH-20. Two ml fractions of the column effluent were collected and their fluorescence determined in an Aminco Bowman spectrophotofluorometer at the predetermined wavelengths for maximum activation (350 nm) and emission (440 nm). Standardization was with 0.1 μg/ml quinine sulfate. Because we encountered spurious fluorescent peaks which were traced to the water used in washing the extracts, and which persisted even with glass-distilled or ion-exchange-treated distilled water, we modified the procedure by reducing the number of washings to two with one-third volume of water each time. This eliminated the spurious peaks which emerged from the Sephadex column. In addition to these two procedures for measuring lipid soluble fluorescence, the procedure of Flectcher et al. (5) was also used. This is similar to the above modified procedure but has only one equal volume water wash of the chloroform-methanol extract.

RESULTS

Lipofuscin Deposition in Uterus

At 2 months the uteri of all rats had the normal white appearance. After 5 months the uteri from vitamin E deficient animals had become brown, while those from controls remained unpigmented. Figure 1 compares one horn of a control uterus (center) flanked by uteri from rats deficient in vitamin E for 5 months (experiment 3). Although the control uterus was white in visible light (Fig. 1A) and non-autofluorescent in ultraviolet light (Fig. 1B), the uteri of vitamin E deficient rats had accumulated pigment which appeared brown in visible light (Fig. 1A) and autofluoresced bright yellow when excited by ultraviolet light (Fig. 1B). No differences in pigmentation or in autofluorescence were seen whether the rats had received retinol (left uterus of Fig. 1A and 1B) or had received retinoic acid instead (right uterus). Likewise, rats fed 10-fold different amounts of retinol (0.8 and 8.0 mg/kg of diet) showed no visible differences in uterine pigmentation or autofluorescence even after 8 months, when all vitamin E deficient uteri had become dark brown. The low dietary retinol content (0.8 mg/kg) provided barely enough of the vitamin to promote a normal growth rate and gave little liver storage, whereas the high level (8.0 mg/kg) gave very high storage (16 μg/g vs. 538 μg/g, respectively, for 3 rats after 8 months). Differences in plasma retinol levels, however, were not so great (23 vs. 34 μg/dl, respectively).

Plasma and liver retinol in the retinoic acid-fed rats were undetectable at 5 months. It should be noted that retinoic acid does not accumulate in any tissue, including liver, and has a rapid turnover (6). In none of the three experiments was there evidence that the presence or absence of dietary retinol had any effect on the amount of lipofuscin in the uterus as determined by gross examination or by ultraviolet autofluorescence of histological preparations (Fig. 2). The number of lipofuscin granules counted in the muscle cells of the uterine wall corresponded to the intensity of fluorescence. All vitamin E deficient rats after 5 and 8 months showed more than a 100-fold increase in number of lipofuscin granules over E-adequate rats, regardless of vitamin A status. Control rats receiving dietary α-tocopherol with low, high or no retinol (retinoic acid-supplemented) had normal appearing uteri with little histological fluorescence and very few lipofuscin granules.

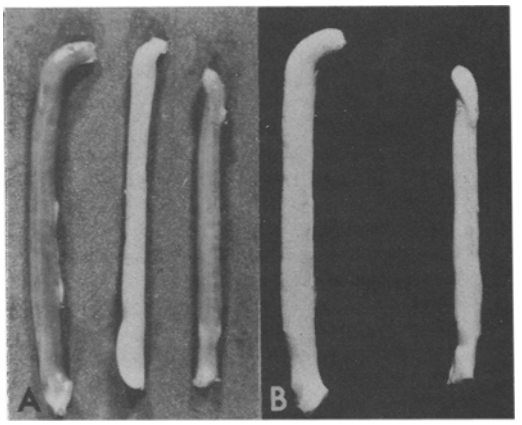

FIG. 1. Left horns of uteri from rats killed after 5 months on a control diet (center of each micrograph), or on vitamin E free diets with 2.0 mg retinol/kg of diet (left) and no retinol but 4.0 mg retinoic acid/kg of diet (right), photographed under white light (Fig. 1A), and under light of ca. 360 nm (Fig. 1B) to elicit autofluorescence of lipofuscin pigment. The control uterus appeared white to light pink in visible light and nonfluorescent in the ultraviolet light. Both vitamin E deficient uteri appeared brown in visible light and bright yellow in ultraviolet light. (X1.4).

Extractable Fluorescence in Uterus

Initial analyses of lipid extracts of uteri (and other tissues) by Sephadex chromatography according to Csallany and Ayaz (4) gave a fluorescent peak, designated A, in column fractions 10-16 which these investigators attributed to lipofuscin. Several other peaks usually appeared in later fractions. In contrast to expectation, however, in the first experiment the primary peak A from control vitamin

E-adequate uteri was of similar magnitude to that obtained from vitamin E-deficient uteri, even though the latter were heavily pigmented. Control extracts with reagents only revealed that much of peak A, and most of the later peaks, were artifacts from the large volume of water used to wash the chloroform-methanol. This water had been distilled and passed through an ion exchange column. A subsequent pass through activated charcoal eliminated most of the fluorescence after fraction 20 and greatly reduced the fluorescence in peak A. To further reduce this artifactual fluorescence, we modified the procedure by greatly reducing the volume of water to two washes of 6 ml each. With this modification, only peak A appeared in E-deficient tissues and either a small or no peak was found in E-sufficient tissues.

In the second experiment, retinol and retinoic acid were fed with and without vitamin E for 14-16 weeks. The uterine extracts were washed only twice with small volumes of water, as described above, prior to being chromatographed on Sephadex. In Table I are shown the relative fluorescence of the whole chloroform extracts and also of peak A from the Sephadex column. There was no significant difference in the extractable fluorescence, either in the whole extract or in the primary peak A, from uteri of E-deficient rats fed retinol or markedly depleted of retinol (maintained on retinoic acid). The fluorescence of the total extracts and also of the Sephadex peaks A for both E-deficient groups were significantly greater than for the E-adequate groups ($p<0.05$). Thus, there does not appear to be any advantage in chromatographing the total lipid extract in order to distinguish between tissues deficient or adequate in vitamin E. The exception may be liver, where the presence of vitamin A can give excessive fluorescence (4). A noteworthy observation in this experiment is that even though counts from histological preparations of uteri would indicate a 100-fold difference or more in lipofuscin pigment granules between E-deficient and E-adequate animals (Fig. 2), the lipid soluble fluorescence indicates only a 2- to 3-fold difference. It is obvious that only a very small fraction of the pigment is extractable, an observation in agreement with the historical definition of this material (7).

A small but detectable amount of retinol was found in rats fed retinoic acid with vitamin E, but no retinol was detected in rats without vitamin E. This confirms the well-known sparing action of α-tocopherol on tissue vitamin A stores (Table I).

In the third experiment, uterine extracts were extracted by the procedure of Fletcher et al. (5) and the relative fluorescence of both the chloroform and water phases determined. As noted by Desai et al. (8), the water phase of extracts from vitamin E-deficient uteri had more fluorescence than the chloroform phase (data not shown). As in experiment two, there was no difference in relative fluorescence whether the rats were fed no retinol (retinoic acid) or adequate retinol.

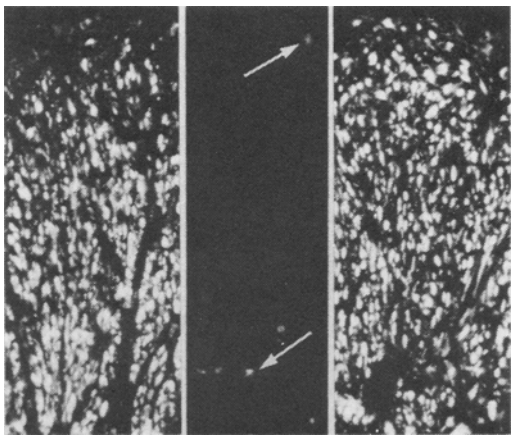

FIG. 2. Lipofuscin fluorescence of frozen transections from the outer (longitudinal) muscle wall of the uteri that are shown grossly in Figure 1. Only a few lipofuscin deposits (arrows) are seen in the control tissue. (X170).

DISCUSSION

The primary purpose of this study was to determine if the vitamin A status of rats could affect the degree of lipofuscin formation in vitamin E-deficient rats. The rationale was a report by Wolfe et al. (2) that the abnormal lipid in nerve tissue from a patient with Batten's disease had a component tentatively identified as a retinoyl complex. In this disease, nerve tissue accumulates an autofluorescent pigment with the characteristics of lipofuscin or ceroid. We selected the uterus as a model because this organ is the most susceptible to lipofuscin accumulation in vitamin E-deficient rats. Under our conditions of depleting weanling rats, the onset of lipofuscin, as noted by gross visual examination or by histological autofluorescence, began after three to four months. By these two criteria and also by measuring the extractable fluorescence, we found no evidence that vitamin A status affected lipofuscin accumulation. Vitamin A status varied widely in the different experiments, from essentially total depletion of

TABLE I

Relative Fluorescence of Organic Solvent Soluble Fluorescent Material from Rat Uterus

Diet[c]	No. rats	Relative fluorescence[a]		Tissue retinol[b]	
		Total extract[d]	Sephadex peak[e]	Plasma μg/dl	Liver μg/g
- Vit. E, + retinol	8	9.7 ± 0.8[1]	1.5 ± 0.3[1]	26.8 ± 8.2	154 ± 8
- Vit. E, + retinoic acid	8	10.1 ± 2.3[1]	1.8 ± 0.3[1]	0	0
+ Vit. E, + retinol	6	2.0 ± 0.5[2]	0.7 ± 0.2[2]	40.3 ± 6.1	186 ± 19
+ Vit. E, + retinoic acid	5	4.1 ± 1.3[2]	0.7 ± 0.2[2]	2.8 ± 2.2	8.8 ± 4.6

[a]Tissues extracted with $CHCL_3$/MeOH and the extract washed twice with water as described in Methods. Values are means ± SEM of relative fluorescence per g tissue after 14-16 weeks. Values in same column with different superscript are significantly different ($P<0.05$).

[b]Values are means ± SEM.

[c]Supplements to the vitamin E deficient diet were: retinol, 2 mg/kg; retinoic acid, 4 mg/kg; α-tocopheryl acetate, 250 mg/kg.

[d]Chloroform extract before chromatography.

[e]Peak A, fractions 10-16, from Sephadex column chromatography of the chloroform extract.

retinol by two months when retinoic acid was fed, to low and high tissue levels as determined from plasma and liver analyses. The uterus is not a storage organ for retinol and the very low content which must be present from circulating blood is not detectable by usual analytical methodology.

After this study was begun, Nelson and Halley (9) questioned the interpretation of the data of Wolfe et al. (2) and suggested that the physical evidence indicated that cholesterol rather than some form of retinol could have generated the data. This controversy has not been resolved to date.

Another aspect of our study was to determine if the amount of organic soluble fluorescence obtained from uterus correlated with the degree of lipofuscin pigmentation seen visually or by histological fluorescence. Although normal uteri from rats fed vitamin E had less extractable fluorescence than did E-deficient uteri, within any group of the latter there was a 10-fold range in extractable fluorescence even though the tissues appeared to have similar pigmentation. Even after chromatography on Sephadex, the peak reported to be lipofuscin (4) varied 5-fold in both normal and E-deficient tissues. As noted by Desai et al. (8) in analyses of E-deficient uteri, there was more fluorescence in the water phase obtained from a chloroform-methanol extract than in the organic phase. Neither phase, however, indicated the magnitude of the difference in pigmentation between E-adequate and E-deficient uteri. For quantitative studies of lipofuscin formation in this tissue, extracts appear to have limited value.

As a corollary of this work, the formation of lipofuscin-like granules in the pigment epithelium of the retina was examined. In this tissue, the observed relationship between vitamins A and E was more complex than in the uterus (10), probably because of the central role of retinol in the visual process.

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Distribution of Phosphoinositides among Subfractions of Rat Brain Myelin

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ABSTRACT

Rat brain myelin was separated into three subfractions, heavy, medium, and light, and the concentrations of phosphatidic acids (PA), phosphatidylinositol (PI), di- (DPI), and triphosphoinositide (TPI) in these fractions were determined. PI was evenly distributed among the fractions, and PA, DPI, and TPI occurred in highest concentrations in the "light" myelin. This result indicates that these fast metabolizing lipids play an important role in the tightly packed central lamellae of the myelin sheath.

INTRODUCTION

The polyphosphoinositides, phosphatidylinositol-4-phosphate (diphosphoinositide, DPI), and phosphatidylinositol-4,5-bisphosphate (triphosphoinositide, TPI), are minor lipid constituents of most eukaryotic tissues. They are of special biochemical interest because the turnover rates of the monoesterified phosphates of the DPI and TPI are the fastest such rates known for any lipid, and they appear to be responsive to physiological stimuli applied to the tissues (1-5).

Polyphosphoinositides of the brain are predominantly associated with the myelin fraction (6-8). Because of the slow turnover of the lipids and proteins of the myelin structure, it has been suggested that the highly reactive polyphosphoinositides, and the necessary enzymes, might be located in myelin appurtenances such as the internal or external mesaxon or the membrane loops at the nodes of Ranvier, i.e., in the loosely packed myelin adjacent to glial cytoplasm, axolemma, or extracellular space (6,7,9,10). We have reported in vitro studies on the biosynthesis of polyphosphoinositides in three myelin subfractions described and characterized by other workers (11-13): "heavy myelin," "medium myelin," and "light myelin," of which the "heavy" fraction contains the most protein and the least lipid, the "light" fraction the most lipid. Heavy myelin consists largely of single lamellae, the light myelin of the typical stacked lamellae of myelin. Thus, the light myelin may be assumed to represent the tightly packed middle layers of the myelin sheath, while the heavy fraction contains much appurtenances, with the medium fraction intermediate in composition. The most active incorporation of ^{32}P from γ-^{32}P-APT into DPI and TPI occurred in the heavy myelin fraction, indicating the association of kinases with the myelin appurtenant structures (10). However, the medium and light myelin also showed appreciable activity, with specific activities (per mg protein) only ca. 50% lower than those of the heavy fraction. This unexpected finding suggests that there is rapid metabolism of phosphoinositides in the core (the most closely packed layers) of the myelin sheaths, and that this region contains the necessary phosphorylating and dephosphorylating enzymes together with their substrates. As part of an investigation of this possibility, we report here the analyses of the distribution of the phosphoinositides, and also of their rapidly metabolizing precursor, phosphatidic acid, in the three subfractions of myelin. We have made use of new methods developed by Eichberg and Hauser (7) which have greatly enhanced recoveries of the polyphosphoinositides in the subcellular fractions, and by Schact (14) who has recently reported a rapid and quantitative purification of DPI and TPI based on the specific affinity of neomycin to the polyphosphoinositides.

MATERIALS AND METHODS

Materials

Rats (25-30 days old) were obtained from Charles River Breeding Labs (Wilmington, MA). Carrier-free (^{32}P) orthophosphate was purchased from ICN (Irvine, CA). DPI and TPI standards, and neomycin sulfate, were from Sigma (Boston, MA). Glycophase-CPG was purchased from Pierce (Rockford, IL).

Isolation and Subfractionation of CNS Myelin

Rats were injected with 25 μl of [^{32}P]-orthophosphate in saline (100 μCi per animal), into a cerebral hemisphere. Ten minutes later the animals were killed by decapitation and the brains were quickly dissected out and immediately homogenized in 9 vol of a medium containing 0.32 M sucrose in 25 mM Tris-buffer, pH 9.5 (7,15). The homogenates were

then diluted with an equal volume of the medium. Pure myelin (whole myelin) and the subfractions (light myelin, 0.32-0.5 M interface; medium myelin, 0.5-0.63 M interface; heavy myelin, 0.63-0.73 M interface; pellets, bottom of 0.73 M) of the myelin were then isolated by centrifugation as reported earlier (10,11), except that all the sucrose solutions used for making the gradient were prepared in 25 mM Tris-buffer, pH 9.5, and the distilled water was replaced with the buffer (without sucrose) for the osmotic-shock step in the preparation of total myelin. Brain homogenates from 3 animals were pooled for each experiment to prepare the myelin subfractions.

Extraction of Phospholipids

The washed and pelleted fractions (10) were homogenized in chloroform/methanol (1:2, v/v; 5 ml per g original brain) with a Tekmar Tissuemizer (SDT-182EN, Tekmar, Cincinnati, OH). The lipids were then extracted after addition of 0.6 ml of 1 M KCl and 0.6 ml of chloroform per 1 ml of the homogenates. The tissue cake at the interphase was re-extracted once with chloroform/methanol/1.2 N HCl (2:1:1, v/v) (16). The rest of the extraction procedure was as described by Schact (14). The extractions were carried out on the day of fractionation. The lipid extracts were dried under N_2 and stored at -20 C overnight.

Isolation of TPI and DPI by Column Chromatography

TPI and DPI were separated from the rest of the lipids by chromatography on immobilized neomycin columns with the following modifications of the original procedure (14). Total lipid extracts fo the fractions were applied to 1 ml of immobilized neomycin columns. The phospholipids other than polyphosphoinositides (fraction I) were quantitatively eluted with 8 ml of 150 mM ammonium acetate in chloroform/methanol/water (3:6:1, v/v/v). DPI was then eluted with 8 ml of 600 mM ammonium acetate in the same solvent, and TPI with 16 ml of chloroform/methanol/conc. ammonia (3:6:1, v/v/v). The columns were finally washed with chloroform/methanol/conc. HCl (3:6:1, v/v/v) to assure the complete recovery of TPI. The last few drops of the eluates of each fraction were routinely monitored for [^{32}P] radioactivity to check the complete resolution of the column fractions. After elution, the fractions were either used directly for the radioactivity, phosphorus determinations and thin layer chromatography, or acidified, and the lipids washed free of salt and dried under N_2 prior to storage.

Thin Layer Chromatography

To check their purity the lipids were dissolved in chloform/methanol/H_2O (75:25:2, v/v/v), and applied to precoated TLC plates of Silica Gel 60 (EM Lab., Elmsford, NY). The plates were developed in chloroform/methanol/conc. ammonia/water (90:90:7:22, v/v) (14). Phosphatidylinositol (PI) and phosphatidic acid (PA) present in column fraction I were separated by two dimensional thin layer chromatography on Silica Gel H magnesium acetate impregnated plates (17) which were developed in chloroform/methanol/28% ammonia (70:30:6, v/v/v) in the first dimension, and in chloroform/acetone/methanol/glacial acetic acid/water (5:2:1:1:0.5, v/v) in the second. The lipid spots on the plate were located by scanning for radioactivity (Berthold scanner LB2760, Shandon, Sewickley, PA), and exposure to iodine vapors.

Analytical

Total phosphorus of lipid samples in column fractions and TLC spots was determined by the method of Bartlett (18) after digestion of the samples with acid mixture (85% sulfuric acid/72% perchloric acid, 2:1, v/v). Counting of radioactivity and assay procedures of the marker enzymes were done as reported earlier (10).

RESULTS AND DISCUSSION

Table I shows the efficacy of the alkaline medium in regard to the protection of the polyphosphoinositide against breakdown during subcellular fractionation. The radioactive label served as check on the purity and also as check on the recoveries of the isolated polyphosphoinositides. Two groups of two rats each (25 days old) were injected intracerebrally with ^{32}P-orthophosphate, and the brains were removed 10 min after the injection. The brains of one group were immediately frozen in liquid N_2, the brains of the other group were rapidly homogenized (individually) in 10 ml of 0.32 M sucrose in pH 9.5 Tris-buffer, and the homogenates were kept at 4 C for 4 hr (approximate time for brain fractionation) prior to the extraction, separation, and quantitation of the lipids. There is good agreement between the two groups with respect to the recoveries of the radioactivity and the quantities of the phospholipids found in each of the fractions. Thus, the addition of alkaline Tris-buffer to the homogenization medium effectively preserves DPI and TPI.

Purity of the column fractions was determined by thin layer chromatography. Radio-

chromatography of the fractions as well as staining of the plates with phosphorus reagent showed only one major spot in the column fractions containing DPI and TPI. Column fraction I containing total phospholipids minus polyphosphoinositides showed one major broad radioactivity peak under these conditions of separation. Further fractionation by two dimensional thin layer chromatography revealed that ca. 70% of the radioactivity was associated with phosphatidic acid.

The distribution of marker enzymes (Table II), in close agreement with published results of myelin fractionation (10,11), show that the presence of pH 9.5 buffer during the preparation of the myelin and its subfractions did not alter the degree of purification. The specific myelin marker, 2',3' cyclic AMP phosphohydrolase, was enriched about 4-fold (over the homogenate) in myelin and its subfractions. Activities of 5'nucleotidase and ATPase (tpyical plasma membrane markers), LDH (supernatant marker), NADPH cytochrome-c reductase (microsomal marker), and succinic-INT reductase (mitochondrial marker) are low in all the fractions (Table II). The recovery of whole myelin ATPase in the subfractions is low possibly because of removal of neuronal, synaptic and axonal plasma membranes (with high ATPase activity) contaminating the whole myelin fraction. LDH recoveries may appear high because some soluble cytoplasm remains entrapped in myelin vesicles during centrifugations, and is then released during the assay by the action of Triton-X-100. However, LDH activity in whole myelin is only 1% of that of

TABLE I

Prevention of the Degradation of Polyphosphoinositides of Brain Homogenates by Alkaline Medium[a]

Conditions of the experiment	Column Fractions			
	DPI		TPI	
	μm[b]	cpm x 10^6	μm	cpm x 10^6
Brains frozen immediately	0.21 (0.17-0.25)	0.84 (0.73-0.94)	0.31 (0.29-0.33)	0.90 (0.87-0.93)
Brain homogenates in alkaline medium kept at 4 C for 4 hr	0.22 (0.16-0.27)	0.92 (0.91-0.93)	0.36 (0.24-0.48)	1.33 (1.24-1.42)

[a]Experimental conditions as detailed in Results. Extraction, separation and quantitation of the lipids as described in Methods. Values are averaged from the two individual values in parentheses.

[b]μm - μmoles and radioactivity of the lipid per g wet wt of brain.

TABLE II

Percent of Whole Homogenate Activities of Marker Enzymes in the Subfractions of Myelin Prepared with the Modified Conditions of Fractionation[a]

Fractions	2',3'-Cyclic AMP phosphohydrolase*	5'-Nucleotidase	ATPase	LDH[b]	NADPH cyt.-c reductase	Succinic-INT reductase
Whole myelin	27.5	4.9	2.9	1.11	2.84	<0.01
Light myelin	1.9	0.37	0.09	0.12	0.18	<0.01
Medium myelin	10.2	1.58	0.74	0.73	0.88	<0.01
Heavy myelin	7.5	0.93	0.11	<0.01	0.27	<0.01
Pellet	1.6	0.43	0.06	<0.01	0.11	<0.01

[a]Experimental conditions as described in "Methods." 2',3'cyclic AMP phosphohydrolase assayed according to Sims and Carnegie (21).

[b]Lactate dehydrogenase (LDH) was assayed in the presence of optimal amounts of Triton-X-100.

TABLE III

Distribution of Polyphosphoinositides in the Myelin Subfractions, Per Gram of Wet Rat Brain[a]

Fractions	TPL	PI		PA		DPI		TPI	
	μmole	μmole	%[b]	mole	%[b]	nmole	%[b]	nmole	%[b]
Homogenates	41.03	1.26	3.1	0.47	1.15	160	0.4	267	0.65
Whole myelin	10.01	0.27	2.7	0.22	2.2	70	0.7	147	1.47
Light myelin	1.39	0.049	3.53	0.067	4.8	18	1.3	24	1.73
Medium myelin	3.11	0.096	3.09	0.061	1.96	19	0.61	41	1.32
Heavy myelin	1.72	0.055	3.2	0.034	1.98	5	0.3	22	1.28
Pellet	0.49	0.014	2.86	0.01	2.04	2	0.41	3	0.61
Recoveries(%)[c]	67 (61-74)	79 (68-82)	---	78 (65-85)	---	63 (60-71)	---	61 (59-68)	---

[a]The phospholipids of the fractions were extracted and separated as described in Methods. Values are averages of 3 to 5 fractionations. TPL – total phospholipids; PA – phosphatidic acid; PI – phosphatidylinositol; DPI – diphosphoinositide; TPI – phosphatidylinositol-4,5-bisphosphate.

[b]Mole percent of total phospholipids of the fraction.

[c]Percent of whole myelin phospholipids recovered in all subfractions of myelin. Values in the parentheses represent the range of recoveries.

whole brain. So, contamination of myelin fractions by cytoplasm is very low.

The results of the phospholipid analyses of the fractions are given in Table III. Recoveries of whole myelin phospholipids in all of the subfractions of myelin taken together ranged from 61 to 79% and were in the range of total protein recoveries (68%). The loss of up to 40% of polyphosphoinositides is, therefore, probably caused by loss of total myelin membranes during subfractionation and purification, though action of hydrolases or adsorption of DPI and TPI on contaminating membranes may not be completely excluded.

The concentrations of the phospholipids in the brain homogenates and whole myelin are in general agreement with values reported by others (7,19,20). PA, DPI, and TPI concentrations of whole myelin, expressed as mole percent of total phospholipids, are about 2-fold enriched over the homogenate, while the percentages of PI are similar in both these preparations. PI is also evenly distributed over the three myelin subfractions. However, PA (the precursor of the inositides), DPI and TPI concentrations in light myelin are substantially higher than those in medium and heavy myelin; DPI is also more concentrated in medium than in heavy myelin.

These results show that, although the polyphosphoinositides are present in that subfraction of myelin which is rich in myelin appurtenances, even higher concentrations are found in those subfractions that represent the packed core of the myelin sheath. This distribution contrasts with that of 2′,3′-cyclic AMP phosphohydrolase but is parallel to that of the myelin basic protein (13). Interestingly, basic protein and polyphosphoinositides associate readily in vitro (22). The phosphohydrolase, although considered a specific myelin marker, is more concentrated in the heavier fractions of myelin (13,23,24), i.e., in the appurtenant elements of myelin; it has been proposed that this enzyme is involved in the formation, rather than the function, of myelin (13). Basic protein, on the other hand, is undoubtedly a building block of myelin, and, interestingly, it also undergoes rapid phosphorylation (25). We are confronted with the question of what function the fast metabolizing protein and the inositol lipids might have in a structure that is supposed to serve only as an electrical insulator.

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Partial Synthesis of [1-^{14}C]Phytanic Acid

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ABSTRACT

[1-^{14}C]Phytanic acid has been prepared in good yield from the unlabeled acid. Pristanyl iodide, prepared from the latter by a modified Hunsdieker reaction, is converted to the corresponding [^{14}C]-nitrile by reaction with sodium [^{14}C]cyanide in dimethyl sulphoxide; hydrolysis of the [^{14}C]nitrile yields [1-^{14}C]phytanic acid. The labeled acid should prove to be a useful substrate for the diagnosis of Refsum's disease.

INTRODUCTION

Phytanic acid (3,7,11,15-tetramethyl hexadecanoic acid), is a naturally occurring compound found in trace amounts in most animal tissues (1). In Refsum's disease, it accumulates in tissues due to an inherited deficiency in the mitochondrial enzyme complex, phytanic acid oxidase (2-4). The disorder can be diagnosed biochemically by demonstrating greatly elevated values of phytanic acid in plasma. Confirmation of the diagnosis can be obtained by measuring the activity of the oxidase in skin fibroblasts in culture (3,5). For this determination, [U-^{14}C]phytanic acid is added to the culture medium, and the activity of the cells is assessed by measuring the release of $^{14}CO_2$ from the labeled phytanate.

Preparation of the labeled fatty acid is tedious procedure as it involves growing algae in the presence of ^{14}C-bicarbonate, isolating [U-^{14}C]phytol (6) and then converting it to [U-^{14}C]phytanic acid chemically. Moreover, the yield is low (from ^{14}C-bicarbonate), and because the compound is uniformly-labeled, data obtained may be prone to a certain degree of ambiguity.

In this communication, we describe the partial synthesis of [1-^{14}C]phytanic acid starting from the unlabeled acid. Pristanyl iodide, which is prepared from the latter and by a modified Hunsdiecker reaction, is converted to the corresponding [^{14}C]nitrile. Pristanyl [^{14}C]nitrile is then easily hydrolyzed in high yield to [1-^{14}C]phytanic acid.

MATERIALS AND METHODS

Phytol was purchased from the Aldrich Chemical Co., (Milwaukee, WI). Phytanic acid was prepared from phytol as described by Jellum et al. (7). [^{14}C]Sodium cyanide (60 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, U.K.).

Gas Chromatography

Analyses were performed with a Varian 2740 gas chromatograph fitted with a 1.5 m x 2 mm id stainless steel column of 7% EGS – 1.5% SE 30 on Gas Chrom Q (100/200 mesh); column temperature, 160 C, nitrogen flow, 20 ml/min.

Mass Spectrometry and Nuclear Magnetic Spectroscopy

Electron impact-mass spectrometry (EI-MS) was performed with a Finnigan 4021 quadrupole instrument, using a solid probe inlet, or with a Hewlett Packard 5985A quadrupole machine. The [^{14}C] enrichment of [1-^{14}C]phytanic acid was determined on an AEI MS30 double focusing instrument with single stage jet inlet operating in the SIM mode. Nuclear magnetic spectrometry was performed on a Varian T60 instrument operating at 60 mH.

EXPERIMENTAL

1-Iodo-2,6,10,14-Tetramethyl Pentadecane (Pristanyl Iodide)

A solution of iodine (254 mg, 1 mmol) in 5 ml dry carbon tetrachloride was added to a suspension of red mercuric oxide (250 mg, 1.1 mmol) and phytanic acid (312 mg, 1 mmol) in 10 ml carbon tetrachloride. The mixture was boiled under reflux for 1 hr under an atmosphere of nitrogen, cooled, filtered and washed twice with 4% (w/v) aqueous sodium hydroxide. The carbon tetrachloride solution was then washed twice with water, dried with anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator at 40 C. The residue, dissolved in 10 ml petroleum ether (bp 40-60 C) was applied to a column of silicic acid (10 g, 2 cm diameter) which had been equilibrated with the same solvent. A small amount of nonpolar material was eluted with 25 ml petroleum ether. Elution with a further 100 ml petroleum ether and evaporation of the eluate in a rotary evaporator at 40 C yielded pristanyl iodide (240 mg, 61% yield) as a colorless liquid, bp 115-120 C (0.1 mm). IR (film) ν_{max} 2970, 2940, 2860, 1465, 1380, 1195 cm^{-1}. NMR

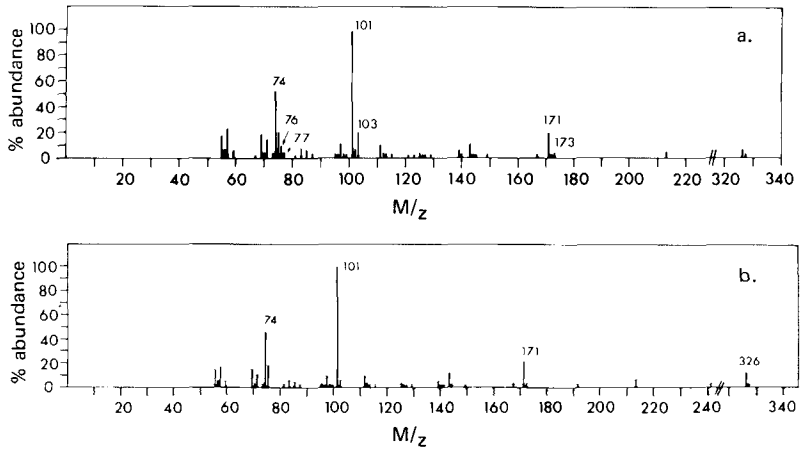

FIG. 1. EI-MS of labelled and unlabelled phytanic acid. EI-MS was performed as described in the text: (a) [1-^{14}C] phytanic acid; (b) unlabelled phytanic acid.

(carbon tetrachloride solution) 3.2 p.p.m. (d, 2H, J = 4Hz, CH_2I). EI-MS M/z 394 (M^+ 0.1%), 267 ($(M - 127)^+$ 1.3%), 57 (100%).

1-[^{14}C] Cyano-2,6,10,14-tetramethyl pentadecane (Pristanyl [^{14}C] nitrile)

Forty milligrams of pristanyl iodide (0.1 mmol) and 6.8 mg sodium [^{14}C] cyanide (0.14 mmol, 1mCi) in 5 ml dry dimethyl sulphoxide were heated at 70 C for 3 hr. The mixture was cooled, extracted three times with 5 ml with hexane and the combined extracts evaporated in a rotary evaporator at 40 C. The residue was dissolved in 2 ml hexane and applied to a column of silicic acid (10 g, 1 cm diameter) previously equilibrated with hexane. Unreacted pristanyl iodide was eluted with 10 ml hexane/ether (4:1, v/v). Elution with further 10 ml hexane/ether (4:1, v/v) yielded pristanyl [^{14}C] nitrile (24.7 mg, 84.5% yield from pristanyl iodide, 6.9 Ci/μmol) as a colorless liquid, bp 105-107 C (0.2 mm); IR (film) ν_{max} 2260 cm^{-1} (CN); EI-MS $M/_z$ 293 (M^+, 3.5%), 71 (100%).

3,7,11,15-Tetramethyl hexadecan-1-[^{14}C] oic Acid ([1-^{14}C] Phytanic Acid)

14.8 mg pristanyl [^{14}C] nitrile, 1.5 ml of 2 mmol/l aqueous sodium hydroxide and 1.5 ml ethanol were heated at 100 C for 16 hr. The mixture was cooled, acidified with aqueous 2N hydrochloric acid and extracted twice with 5 ml hexane. The combined extracts were washed with water and the hexane removed in a rotary evaporator at 40 C and the residue dissolved in 4 ml hexane. Aliquots of this solution (1.0 ml) were chromatographed on 0.5 mm thick Silica Gel H plates in hexane/ether/acetic acid (70:30:1, v/v). The acid was located by spotting the unlabeled compound on the edges of the plate, and spraying these sections with 0.1% (w/v) iodine in methanol. [^{14}C] Phytanic acid (13.9 mg, 88% yield from ^{14}C-pristanyl nitrile) was eluted from the silica with 3 x 5 ml chloroform/methanol (1:2, v/v). Its thin layer and gas chromatographic properties were indistinguishable from the natural unlabeled compound and its specific activity was 6.9 μCi/μmol.

RESULTS AND DISCUSSION

The method adopted to introduce a [^{14}C] label at C-1 of phytanic acid involved degradation of phytanic acid to the pristane skeleton followed by [^{14}C] homologation. A number of methods have been used to convert phytanic acid or its derivatives to pristanic acid. Kates et al. (8) oxidized phytene prepared from phytanyl iodide; Sen Gupta and Peters (9) used a Barbier-Wieland degradation reaction to convert methyl phytanate to pristanic acid, and Smith and Boyack (10) prepared oxidized phytene produced by the pyrolysis of dihydrophytyl stearate.

Pristanic acid is, however, a poor starting point for homologation so we chose instead pristanyl iodide. The synthesis of this compound could be achieved in one efficient step by a modified Hunsdiecker reaction (11). By analogy with the preparation of 4,8,12,16-tetramethylheptadecanoic acid from phytanyl iodide (12), we tried a Grignard reaction between pristanyl iodide and $^{14}CO_2$, but without success. It was possible, however, to

convert the iodide to pristanyl [^{14}C] nitrile with sodium [^{14}C] cyanide in dimethyl sulphoxide in relatively good yield. While vigorous conditions were found to be necessary to hydrolyze the nitrile, the yield of [1-^{14}C] phytanic acid was high.

The identity of the final product was confirmed by thin layer chromatography (TLC) of the free acid, and by gas chromatography (GC) of its methyl ester. Based on the latter data, the product was estimated to be at least 98% pure (chemically and radiochemically). EI-MS of the butyldimethylsilyl ether derivative of its reduction product, [^{14}C] dihydrophytol, indicated a 13.9% ^{14}C]-enrichment (cf. Na^{14}CN, 14.1% ^{14}C). The EI-MS spectra of the labeled and natural methyl phytanate are shown in Figure 1. Spectra of both are almost identical, except for the presence of appreciable amounts of m/z 76, 77, 103 adn 173 in the spectrum of the labeled acid. These particular fragments are indicative of the presence of ^{14}C.

The evidence for the exact localization of the radioactive atom in the final product was based largely on the synthetic technique employed. However, the specific radioactivity determinations, allied with the enrichment data, provided further evidence for the exclusive localization of the label in the 1-carbon atom.

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Occurrence of 24(*E*)-Ethylidene Sterols in Two Solanaceae Seed Oils and Rice Bran Oil

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ABSTRACT

The occurrence of two 24(*E*)-ethylidene sterols, fucosterol and 28-isocitrostadienol, in the unsaponifiable matters of two Solanaceae seed oils from *Datura stramonium* and *Capsicum annuum*, and rice bran oil from the seeds of *Oryza sativa* (Gramineae) was demonstrated by their isolation or by gas liquid chromatography. Although *Z*-isomers of the above two 24-ethylidene sterols, 28-isofucosterol and citrostadienol, are a frequent occurrence in higher plant materials including some Solanaceae seed oils and rice bran oil, this report might be the second instance of the unambiguous demonstration of the occurrence of the 24(*E*)-ethylidene sterols in higher plants.

INTRODUCTION

Our previous studies on the sterols of some Solanaceae seed oils (1,2) and rice bran oil (3,4) showed the presence of 24-ethylidene sterols with the *Z*-configuration such as 28-isofucosterol (Scheme I,**Ib**; stigmasta-5,*Z*-24[28]-dien-3β-ol) and citrostadienol (**IIb**, 4α-methyl-5α-stigmasta-7,*Z*-24[28]-dien-3β-ol) among a number of sterols. This paper describes a further study on the sterol constituents of two Solanaceae seed oils from *Datura stramonium L.* and *Capsicum annuum* L., and rice bran oil from the seeds of *Oryza sativa* L. (Gramineae) resulting in the isolation and identification of the *E*-isomers of the above two 24-ethylidene sterols, fucosterol (**Ia**, stigmasta-5,*E*-24[28]-dien-3β-ol) and 28-isocitrostadienol (**IIa**, 4α-methyl-5α-stigmasta-7,*E*-24[28]-dien-3β-ol), besides the two 24(*Z*)-ethylidene sterols, **Ib** and **IIb**.

EXPERIMENTAL

Materials

The 4-desmethyl- and 4-monomethyl-sterol fractions separated from the unsaponifiable matters of *D. stramonium* and *C. annuum* (California chili) seed oils, prepared as described previously (1,2,5), were used in this study. Rice bran was obtained from the seeds of *O. sativa* grown in Fukushima-ken. Authentic specimens of fucosterol (**Ia**) from Prof. N. Ikekawa (Laboratory of Chemistry for Natural Products, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama, 227) and 28-isocitrostadienol (**IIa**) from Prof. W. Sucrow (Gesamthochschule Paderborn, Westfalen, W. Germany), were generously supplied as gifts. 28-Isofucosterol (**Ib**) (1) and citrostadienol (**IIb**) (2) were prepared in this laboratory.

General

Recrystallizations were performed in acetone/methanol. Melting points (mp) taken on a heat block were uncorrected. IR spectra were recorded in KBr on a Type IRA-2, IR spectrophotometer (Japan Spectroscopic Co., Tokyo). ^{1}H-NMR spectra were obtained with a JNM-FX 100 instrument (Japan Electron Optics Laboratory Co., Tokyo) at 100 MHz in deuteriochloroform. The chemical shifts are given in δ with tetramethylsilane as internal standard. Mass spectra (MS, 70 eV) were taken on a Hitachi RMU-7M mass spectrometer with a direct inlet system (Hitachi Ltd., Tokyo). Gas liquid chromatography (GLC) was performed with a Shimadzu GC-4CM instrument (Shimadzu Seisakusho Ltd., Kyoto) equipped with a hydrogen flame ionization detector. Poly I-110 (column 270 C, carrier gas N_2 80 ml/min, retention time of cholesterol acetate ca. 20 min.) and/or OV-17 (column 260 C, carrier gas N_2 60 ml/min, retention time of cholesterol acetate ca. 18 min) SCOT glass capillary column (30 m x 0.3 mm id, Wako Pure Chemical Industries Ltd., Osaka) was used. Argentation (silver nitrate/silica gel, 1:4 w/w) preparative thin layer (0.5 mm thick) chromatography (TLC) was developed 4-6 times with methylene chloride/carbon tetrachloride (1:5, v/v). The RRT on GLC and approximate relative Rf value on argentation TLC for the acetates of authentic sterols were: cholesterol, RRT 1.00 (OV-17), 1.00 (Poly I-110), relative Rf 1.00; **Ia**, RRT 1.72 (OV-17), 1.58 (Poly I-110), relative Rf 0.38; **Ib**, RRT 1.81 (OV-17), 1.67 (Poly I-110), relative Rf 0.35; **IIa**, RRT 2.29 (OV-17), 2.16 (Poly I-110), relative Rf 0.58; and **IIb**, RRT 2.41 (OV-17), 2.28 (Poly I-110), relative Rf 0.52.

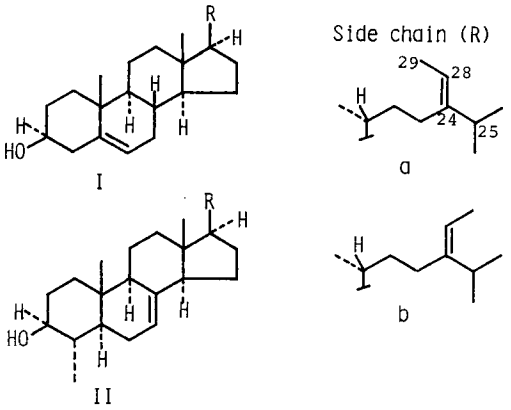
Identification of Fucosterol (Ia) and 28-Isofucosterol (Ib)

As described previously (1), the acetylated 4-desmethylsterol mixture (820 mg) of *D.*

stramonium seed oil afforded 28-isofucosterol (**Ib**) acetate (135 mg, mp 137-138 C) on argentation TLC. The acetate, however, still contained a small amount of an undetermined component (~2% by GLC). In this study, the **Ib**-acetate was, therefore, subjected further to argentation TLC to separate a faint zone slightly less polar than the bulky **Ib** acetate zone. The fraction recovered from this faint zone eventually gave a sterol acetate (3 mg, mp 119-120 C, 93% pure by GLC) after purification by repeated argentation TLC. IR ν_{max} cm^{-1}: 1730, 1250 (OAc), 840, 820, 800 (>C=CH–). MS *m/e* (relative intensity): 394 (M^+-AcOH, 80), 379 (8), 296 (100), 281 (23), 273 (3), 255 (6), 253 (11), 229 (3), 228 (11), 227 (4), 213 (11), 55 (31). ^{1}H-NMR δ: 0.69 (3H, *s*, C-18), 1.01 (3H, *s*, C-19), 2.03 (3H, *s*, C-3β-OAc), 0.98 (3H, *d*, C-21, *J*=6 Hz), 0.98 (6H, *d*, C-26, C-27, *J*=7 Hz), 1.58 (3H, *d*, C-29, *J*=6 Hz), 5.38 (1H, *d*, C-6, *J*=4 Hz), 5.19 (1H, *q*, C-28, *J*=7 Hz), 4.58 (1H, *m*, C-3α, $W_{1/2}$=25 Hz). The sterol acetate was cochromatographed with authentic **Ia** acetate on argentation TLC and GLC. Its mp and spectra were identical with those of authentic **Ia** acetate (mp 119-120 C). The compound was, therefore, identified as **Ia** acetate.

The acetylated 4-desmethylsterol mixture (1.7 g) of *C. annuum* seed oil was separated into seven zones by argentation TLC. A fraction (159 mg) from the fifth zone from the solvent front eventually afforded **Ia** acetate (10 mg, mp 117-119 C) after repeated argentation TLC. The identification was based on chromatographic mobility, IR, MS and ^{1}H-NMR data. **Ib** Acetate (42 mg, mp 137-139 C) also was isolated from the fraction after repeated argentation TLC. IR ν_{max} cm^{-1}: 1720, 1240 (OAc); 805, 795 (>C=CH–). MS *m/e* (rel. int.): 394 (M^+ – AcOH, 40), 379 (5), 296 (100), 281 (13), 273 (2), 255 (3), 253 (6), 229 (3), 228 (7), 227 (3), 213 (9), 55 (12). ^{1}H-NMR δ: 0.68 (3H, *s*, C-18), 1.01 (3H, *s*, C-19), 2.03 (3H, *s*, C-3β-OAc), 0.95 (3H, *d*, C-21, *J*=6 Hz), 0.97 (6H, *d*, C-26, C-27, *J*=7 Hz), 1.59 (3H, *d*, C-29, *J*=6.8 Hz), 5.37 (1H, *d*, C-6, *J*=4.1 Hz), 5.13 (1H, *q*, C-28, *J*=7.1 Hz), 2.83 (1H, *hept.*, C-25, *J*=7 Hz), 4.58 (1H, *m*, C-3α, $W_{1/2}$=22 Hz). These data were identical with those of authentic **Ib** acetate.

Saponification of rice brain oil (745 g), extracted from rice bran (3185 g) with methylene chloride using a Soxhlet extractor, afforded unsaponifiable matter (26 g) which on fractional recrystallization yielded a 4-desmethylsterol fraction. A portion of the 4-desmethylsterol fraction was acetylated with acetic anhydride-pyridine at room temperature overnight, and the resulting acetate fraction (200 mg) was separated into five zones by argentation TLC. GLC on both the Poly I-110 and OV-17 columns of the fraction (10 mg) recovered from the fourth zone from the solvent front indicated evidence for the occurrence of a few percent of **Ia** acetate besides **Ib** acetate, which constitutes the largest portion of the fraction.



SCHEME I. Diagram of skeletons and side chains (R) of sterols.

Identification of 28-Isocitrostadienol (IIa) and Citrostadienol (IIb)

The 4-monomethylsterol fraction (260 mg) of *D. stramonium* seed oil (5), on further TLC in the same way as described previously (2), gave one band which was then divided into halves to give the fraction A (62 mg) from the less polar half and the fraction B (117 mg) from the more polar half. The fraction B was acetylated and the resulting acetate fraction B (110 mg) was separated into six zones by argentation TLC. The fraction (7 mg) recovered from the fifth faint zone from the solvent front eventually afforded a 4-monomethylsterol acetate (~1 mg, ca. 90% pure by GLC, mp 142-145 C) after repeated argentation TLC. IR ν_{max} cm^{-1}: 1730, 1240 (OAc), 822, 818 (>C=CH–). MS *m/e* (rel. int.): 468 (M^+, 10), 453 (10), 393 (7), 370 (56), 355 (6), 327 (100), 310 (15), 302 (8), 295 (8), 287 (6), 269 (26), 267 (22), 243 (9), 242 (10), 241 (13), 227 (28), 55 (73). ^{1}H-NMR δ: 0.54 (3H, *s*, C-18), 0.84 (3H, *s*, C-19), 2.05 (3H, *s*, C-3β-OAc), 0.86 (3H, *d*, C-30, *J*=7 Hz), 0.98 (6H, *d*, C-26, C-27, *J*=6.8 Hz), 0.99 (3H, *d*, C-21, *J*=5.6 Hz), 1.58 (3H, *d*, C-29, *J*=7 Hz), 5.19 (1H, *q*, C-28, *J*=7 Hz), 2.20 (1H, *hept.*, C-25, *J*=6.6 Hz), 4.40 (1H, *m*, C-3α, $W_{1/2}$=25 Hz), 5.19 (1H, *m*, C-7, $W_{1/2}$=20 Hz). Though a

small discrepancy in mp between the unknown sterol acetate and authentic **IIa** acetate (mp 138-141 C; lit. [6], mp 129 C) was observed, the sterol acetate was cochromatographed with authentic **IIa** acetate on argentation TLC and GLC, and the spectra described above were found identical with those recorded for authentic **IIa** acetate. Therefore, the sterol acetate was identified as **IIa** acetate. The sixth zone from the solvent front on the argentation TLC afforded **IIb** acetate (27 mg, mp 149-151 C). IR ν_{max} cm^{-1}: 1720, 1250 (OAc), 830, 815, 800 (>C=CH–). MS *m/e* (rel. int.): 468 (M^+, 4), 453 (4), 393 (6), 379 (48), 355 (6), 327 (100), 310 (20), 302 (7), 295 (10), 287 (4), 269 (15), 267 (34), 243 (6), 242 (10), 241 (10), 227 (20), 55 (58). ^{1}H-NMR δ: 0.54 (3H, *s*, C-18), 0.84 (3H, *s*, C-19), 2.05 (3H, *s*, C-3β-OAc), 0.85 (3H, *d*, C-30, *J*=6 Hz), 0.95 (3H, *d*, C-21, *J*=6.5 Hz), 0.98 (6H, *d*, C-26, C-27, *J*=7 Hz), 1.59 (3H, *d*, C-29, *J*=6 Hz), 5.18 (1H, *d*, C-7, *J*=6.6 Hz), 5.11 (1H, *q*, C-28, *J*=6.6 Hz), 2.83 (1H, *hept.*, C-25, *J*=6.9 Hz), 4.51 (1H, *m*, C-3α, $W_{1/2}$=25 Hz). These data and the chromatographic mobility data of this compound were identical with those of authentic **IIb** acetate.

Fractional recrystallization of the unsaponifiable matter of rice bran oil mentioned above yielded another fraction (5 g) rich in 4-monomethylsterols. This was then subjected to TLC affording a purified 4-monomethylsterol fraction (590 mg). The fraction, on further TLC as described above, gave one band which was then divided into halves to give the fraction A (150 mg) from the less polar half and the fraction B (280 mg) from the more polar half. The fraction B was acetylated and the resulting acetate fraction B (250 mg) was fractionated roughly into three fractions by argentation TLC. The fraction rich in **IIb** acetate from the medium polar zone, on further repeated argentation TLC, eventually afforded **IIa** acetate (2 mg, 91% pure by GLC, mp 141-145 C) and **IIb** acetate (30 mg, mp 148-150 C). The identification of these compounds was confirmed by argentation TLC, GLC, MS and ^{1}H-NMR comparisons with those of the authentic specimens.

The 4-monomethylsterol fraction (279 mg) from the unsaponifiable matter (4.4 g) of *C. annuum* seed oil was further separated into two fractions, A (93 mg) and B (107 mg), by TLC in the same way as described above. The presence of **IIb** acetate as well as a trace amount of **IIa** acetate in the acetylated fraction B (100 mg) was confirmed by GLC on both the Poly I-110 and OV-17 columns.

RESULTS AND DISCUSSION

Because of the close similarity in the mobility data on argentation TLC of 24(*E*)- and 24(*Z*)-ethylidene sterols, and moreover, because the 24(*E*)-ethylidene sterols occur in very minute quantities in the sterol fractions of the plant materials investigated, the isolation of the 24(*E*)-ethylidene sterols could be accomplished with great difficulty in this study. As has already been discussed closely, the 24-ethylidene sterols with 24(*E*)- and 24(*Z*)-configurations were distinguished either by GLC (7-10), IR (10-12), MS (8) or by ^{1}H-NMR (6,13-17) data. Among these data, the ^{1}H-NMR provided especially significant information about the configuration of 24-ethylidene group of sterols by the appearance of C-25 (1H, *heptet*) and C-28 (1H, *quartet*) proton signals: 24(*E*)-ethylidene sterols, **Ia** acetate (C-25, the *heptet* signal which might be expected to appear around δ 2.20, could not be recognized because of the presence of other unassigned proton signals around that field; C-28, δ 5.19), and **IIa** acetate (C-25, δ 2.20; C-28, δ 5.19); 24(*Z*)-ethylidene sterols, **Ib** acetate (C-25, δ 2.83; C-28, δ 5.13) and **IIb** acetate (C-25, δ 2.83; C-28, δ 5.11).

24(*Z*)-Ethylidene sterols such as **Ib**, **IIb** and avenasterol (5α-stigmasta-7,*Z*-24[28]-dien-3β-ol) are now known as widely distributed sterols in higher plants (18-22), whereas 24(*E*)-ethylidene sterol, **Ia**, has been found in marine brown algae (18,23) as the predominant sterol constituent and in a fungus, *Phycomyces blakeleeanus* (24), but has never been found in higher plants with unambiguous evidence for its identification. On the other hand, **IIa**, which has previously been synthesized by Sucrow et al. (6), has never been found in the plant kingdom. Although none of the reports by previous authors (8,19) have indicated the co-occurrence of both *Z* and *E* series compounds in an organism, this study demonstrated unambiguously the co-occurrence of 24(*E*)-ethylidene sterols (**Ia** and **IIa**) and 24(*Z*)-ethylidene sterols (**Ib** and **IIb**), though the former two occur as very minute sterol constituents, in the sterol fractions separated from the two Solanaceae seed oils from *D. stramonium* and *C. annuum* and rice bran oil from the seeds of *O. sativa* (Gramineae). This finding might be significant because sterols with a 24-ethylidene group appear to be important intermediates in the biogenesis of plant sterols (18,20,25).

Though the possibility exists that the *E*-isomer of 24-ethylidene sterols resulted from isomerization of the *Z*-isomer during the extensive separation procedures by argentation

TLC, the 24(*E*)-ethylidene sterols, **Ia** and **IIa**, described here are regarded indeed as the natural products rather than the artefacts produced from **Ib** and **IIb**, respectively, during the separation procedures because preliminary direct GLC as the acetate and trimethylsilyl ether derivatives of the 4-desmethyl- and 4-monomethylsterol fractions of the seed oils examined, prior to the subjection on the argentation TLC, showed the evidence for the presence of **Ia** and **IIa**, respectively, as the minor constituents. GLC indicate that the 24(*E*)-ethylidene sterols, **Ia** and **IIa**, occur further in the seed oils of other Solanaceae described previously (1,2); i.e., *Nicotiana tabacum, Lycopersicon esculentum, Solanum melongena, Physalis alkekengi* var. *francheti* and *Lycium chinense* (T. Itoh, T. Tamura and T. Matsumoto, unpublished results).

Recently, a 24(*E*)-ethylidene sterol, stigmasta-7,*E*-24(28)-dien-3β-ol, has been isolated from the roots of *Bryonia dioica* (Cucurbitaceae) (17), and hence, our study is the second instance for the unambiguous demonstration of the presence of 24-ethylidene sterols with the *E*-configuration in higher plants.

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Microsomal Phosphatidic Acid Phosphohydrolase of Rat Mammary Tissue: I. General Properties

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ABSTRACT

The microsomal bound phosphatidic acid phosphohydrolase from lactating rat mammary tissue had a specific activity of six nmoles per mg protein per minute. The optimum pH was 7.0; magnesium at 1.3 mM was required for maximum activity, and at low substrate concentrations magnesium lowered the Km of the enzyme for phosphatidic acid. Diglycerides exerted little effect while diglyceride ether stimulated enzyme activity. Inorganic salts, i.e., potassium phosphate and potassium chloride, enhanced rates of phosphatidic acid hydrolysis under standard assay conditions.

INTRODUCTION

Milk lipids are composed predominantly of triglycerides (TG) synthesized by the terminal acylation of *sn*-1,2 diglycerides (DG) which are derived from monoglycerides and diacyl *sn*-glycerol-3-phosphate or phosphatidic acid (1-6). Phosphatidic acid (PA) is synthesized in mammary tissue by the sequential acylation of glycerol phosphate, and possibly some dihydroxyacetone phosphate, by microsomal acyl transferases (1,7,8). Some PA is converted to acidic phosphoglycerides via cytidine diphosphodiacylglycerol (9), but most is rapidly dephosphorylated to diglycerides by the enzyme phosphatidate phosphohydrolase (PAPase). Little is known concerning the properties of this enzyme in lactating mammary tissue, though indirect evidence indicates that it is very active (2,5,10). There is some indirect evidence that PAPase may control TG synthesis in liver and adipose tissue (11-13). The possibility that PAPase may be a regulatory step in the synthesis of milk lipids warrants examination. In this initial study the activity and some properties of the microsomal PAPase from rat mammary tissue are described.

MATERIALS AND METHODS

Materials

The sodium salts of dipalmitoyl-*sn*-glycerol-3-phosphate and diacylglycerol-3-phosphate were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H] dipalmitoyl-*sn*-glycerol-3-phosphate was prepared from uniformly labeled [^{3}H] dipalmitoyl-*sn*-glycerol-3 phosphorylcholine (New England Nuclear Corp. Boston, MA) by phospholipase D (Calbiochem Co., Los Angeles, CA) according to the procedure of Kates and Sastry (14). Buffer materials, dithiothreitol (DTT) and various reagent grade chemicals were purchased from Calbiochem Co., (Los Angeles, CA). Chromatographic materials were obtained from Applied Science (State College, PA). The buffers,N,N-bis(2 hydroxyethyl-2-amino enthanesulfonic acid) (BES); N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES); 2[N-morpholino] ethane-sulfonic acid, (MES); N,N-bis-(2-hydroxyethylglycine) (BICINE); and tris-(hydroxymethylaminomethane) (TRIS) were purchased from Serdary Research Co. (London, Ontario). An analog of PA, i.e., DL-2,3-dialkoxypropyl-phosphoric acid, was obtained from Dr. Arthur Rosenthal (Rosenthal and Pousada, 15). The purity of lipid substrates were confirmed by thin layer chromatography (16).

Preparation of Microsomes

Rats (Sprague-Dawley) that were lactating for 8-10 days were killed by cervical dislocation and immediate decapitation. Mammary glands were carefully excised to minimize contamination with adipose, muscle, or connective tissue. All subsequent procedures were performed at 4 C. The mammary tissue was washed with 0.25 M sucrose, blotted dry with cheesecloth and weighed. After chopping with scissors, the tissue was homogenized for 30 sec at high speed in 5 vol of 0.25 M sucrose on a two-speed Waring Blendor drive unit with stainless steel semimicro jar and blade unit accessories (VWR Scientific, Rochester, NY). Tissue that associated upon homogenization was dispersed by chopping with scissors and rehomogenizing for a further 30 sec. This crude homogenate was centrifuged at 15,000 x g for 20 min at 4 C in a Sorval Superspeed RC2-B centrifuge with fixed angle rotor (Sorval type SS-34, r_{av} 4.25"). The supernatant was decanted through four layers of cheesecloth and centrifuged at 44,000 x g for 1 hr at 4 C in a Beckman L2-65 ultracentrifuge with fixed angle rotor (Beckman type 21, r_{av} 9 cm). The microsomal fraction was collected after careful decantation of the high

speed supernatant and suspended in 0.25 M sucrose to 20-30 mg protein/ml using a Ten Broeck tissue homogenizer. After sonication for 1 min in a Model 8845-3 Sonicator (Cole-Palmer Ultrasonic Cleaner), aliquots of the microsomal suspension were analyzed for protein and assayed for phosphohydrolase activity as described below, or it was lyophilized in a Model 10-100 Virtis Unitrap freeze-dryer (VWR Scientific, Rochester, NY) and stored at -20 C.

Preparation of the Microsomal Suspension

When fresh microsomes were used as the enzyme source, the procedure outlined above was employed. With lyophilized microsomes, appropriate amounts were homogenized at 4 C for 1 min in 0.25 M sucrose using a Ten Broeck tissue homogenizer to give a final protein concentration of 20-30 mg/ml. Suspended microsomes were then sonicated for 1 min at 4 C and maintained at that temperature. Protein determination was made prior to each assay by the procedure of Lowry et al. (17) using crystalline bovine serum albumen as standard.

Enzyme Assay

The standard assay contained dithiothreitol DTT (0.6 mM), $MgCl_2$ (1.3 mM), phosphatidic acid, sodium salt (1.3 mM) made up to a final volume of 500 μl with HEPES buffer (0.1 M), pH 7.0' The phosphatidic acid stock solution was dispersed in buffer and sonicated for 1 min at 4 C (Cole-Palmer, Model 8845-3 Sonicator) prior to addition to the assay tubes. The reaction was initiated by the addition of the dispersed microsomes (0.5-0.6 mg protein) and carried out at 35C for specified times using an Evapo-Mix shaker-incubator (Buchler, Fort Lee, NJ). Appropriate zero-time and control assays, i.e., substrate and enzyme blanks, were also run. The reaction was terminated by the addition of 0.4 ml 5% trichloroacetic acid. After cooling to 4 C, the assay tubes were centrifuged for 10 min at 5,000 g to sediment the precipitated protein. Phosphatidic acid phosphohydrolase activity was measured by the release of inorganic orthophosphate, which was quantified in the supernatant by the method of Bartlett (18). A standard curve was constructed using known amounts of potassium phosphate in assay mixtures identical to those used in enzyme assays except heat-denatured microsomes were included. In the absence of enzyme or with heat-inactivated microsomes, no inorganic phosphate was released from the phosphatidic acid.

In experiments with H^3-phosphatidic acid, assay conditions were as described above except the reaction was stopped by extracting the lipids by the method of Folch et al. (19). The lipid classes were then separated by thin layer chromatography (16), located with iodine vapor and, after evaporation of excess iodine, they were transferred to scintillation vials. The radioactivity was determined by liquid scintillation counting in a Packard Model 3385 spectrometer.

RESULTS

Using sonicated dispersions of PA (sodium salt), most of the PAPase activity was associated with the microsomal fraction from lactating rat mammary tissue. The rate of release of Pi was linear with protein concentrations from 0.1 to 1.5 mg per assay for up to 30 min incubation (Fig. 1). Above this concentration, the release of Pi leveled off. This may have been caused by binding of substrate to the protein or by alteration of the physical state of the substrate, i.e., changing from a micellar to a monomeric state as nonspecific binding of substrate to the microsomal protein increased (20,21).

Maximum activity was observed around pH 7.0 with significant activity over the broad pH range from 5.0 to 8.0 (Fig. 2).

The rate of Pi release increased with PA concentration (Fig. 3); however, it did not follow a regular hyperbolic function, but showed a biphasic response around 0.5 mM and maximum velocity at 1.5 mM PA. The break in the V/S curve may have reflected a phase

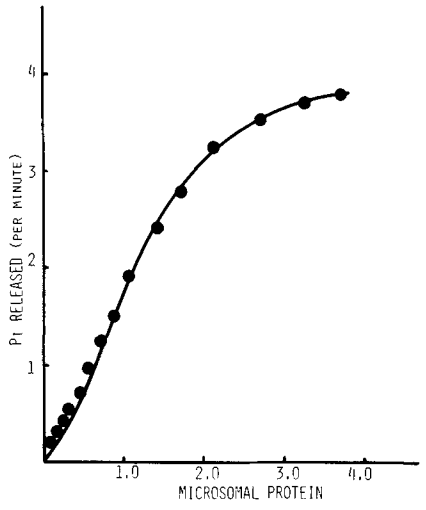

FIG. 1. The effect of protein concentration on the rate of release inorganic phosphate (Pi) from phosphatidic acid by microsomes from rat mammary tissue. Standard assay as described in methods.

transition (monomer to micellar) as the PA concentration was increased. The maximum rates of PA hydrolysis at substrate saturation ranged from 4.4 to 6.1 nmoles/min/mg protein for mammary microsomal preparations from several rats, which incidentally, were significantly lower than the rates (11 ± 3 nmoles) obtained with hepatic microsomes from the same animals. The activity of mammary homogenate was around 0.5 nmole/min/mg of mammary tissue protein.

Though the mammary microsomal PAPase was active in the absence of added magnesium, this cation was necessary and maximum activity was observed around concentrations of 1.5 mM Mg^{2+}. Allowing for the binding of the Mg^{2+} to PA [an association constant of 10,000 was used (22)], the maximum rate was observed at "free" magnesium $(Mg^{2+})_f$ concentrations of 0.4 mM. (Fig. 4) At concentrations greater than this, progressive inhibition occurred.

Experiments were performed to determine the effects of varying levels of non-PA bound, i.e., "free" magnesium $(Mg^{2+})_f$, on the activity of the enzyme. Increasing levels of $(Mg^{2+})_f$ from 0.15 to 1.0 mM, at constant, but varying levels of substrate Mg·PA, increased the rates of hydrolysis, particularly at the lower concentrations of the substrate (Fig. 5A,B). The apparent V did not change, but the apparent Km decreased. At higher concentrations of $(Mg^{2+})_f$, particularly above 1.0 mM, inhibition occurred, especially at low substrate concentrations and the apparent Km was increased while the apparent Vmax decreased. These data suggested that magnesium was an enzyme ligand acting directly on the enzyme altering its affinity for the substrate (Mg·PA). However, the possibility of magnesium changing the physical state of the substrate cannot be ruled out, and at high concentrations magnesium may also have reduced the solubility of the PA substrate.

Because significant amounts of 1,2-diglycerides (DG) are synthesized from 2-monoglycerides in lactating mammary tissue, it is possible that these may affect DG production from PA by inhibiting PAPase. However, the inclusion of 1,2-DG in the assays at concentrations from 0.02-1.5 mM dispersed in Tween 20, exerted negligible effects on PAPase. Because microsomes from lactating mammary tissue contain lipase(s) which hydrolyze 1,2-DG to monoglycerides and fatty acids, we tested the effect of 1,2-diglycerol ether (DGE), which is resistant to lipolysis, at several concentrations up to 1.5 mM, in assays. The DGE actually enhanced the apparent Vmax especially at low substrate levels without altering the apparent Km (Fig. 6). This effect could be attributed to the alteration of the aggregation state of the substrate whereby the DGE acted as a spacer molecule facilitating enzyme substrate interaction. Stimulation of PAPase by amphipathic molecules, e.g., phosphatidylcholine, has been observed by others (23).

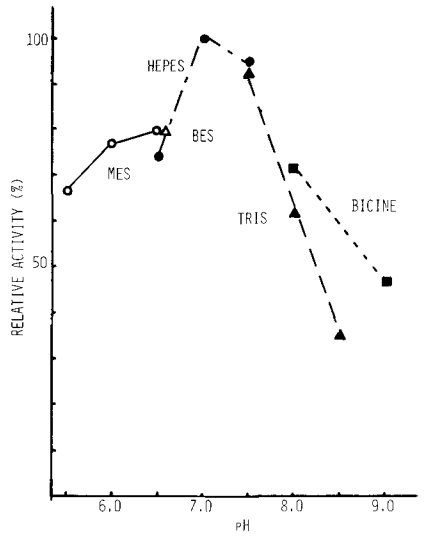

FIG. 2. The effect of pH on the activity of phosphatidic acid phosphohydrolase of rat mammary tissue. Standard assay conditions were used with 2[N-morpholino]ethanesulfonic acid (MES); N,N-bis-(2-hydroxyethyl-2-amino ethanesulfonic acid) (BES); N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); tris-(hydroxymethylaminomethane) (TRIS) and N,N-bis-(2-hydroxyethylglycine) (BICINE) buffers, respectively.

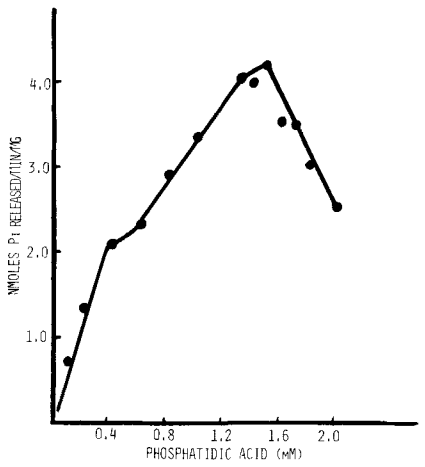

FIG. 3. The effect of substrate concentration on phosphatidic acid phosphohydrolase activity of rat mammary microsomes. Standard assay conditions as described in methods.

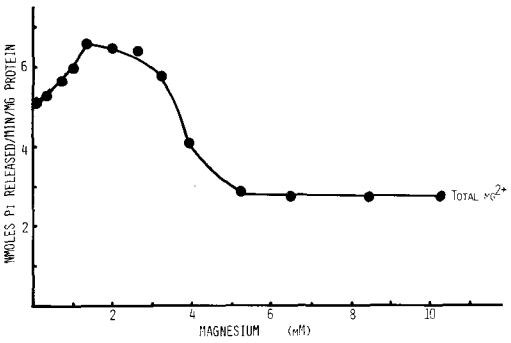

FIG. 4. The effect of total magnesium concentration on the activity of microsomal phosphatidic acid phosphohydrolase of rat mammary tissue.

The inclusion of low concentrations of inorganic phosphate in the assay progressively depressed PAPase activity (Table I). However, at high concentrations, from 0.1 to 0.7 M, phosphate enhanced the rate of hydrolysis of PA. Labeled PA was used in these studies, and the products were mostly 1,2-DG and monoglycerides with small quantities of labeled triglycerides being recovered. The preponderance of the remaining radioactivity was associated with PA.

Because of the influence of inorganic phosphate on the activity of PA phosphohydrolase, we speculated that this might be attributable to the effects of the ionic strength of the assay medium on the substrate or on the enzyme *per se*. Therefore. we examined varying concentrations of potassium chloride (KCl) on PAPase. Potassium chloride acted as a stimulator and inhibitor of the enzyme depending upon the concentration used (Fig. 7). At relatively low concentrations, enhancement of the hydrolysis occurred; whereas, at 1.6 mM, inhibition was observed. The KCl reduced the apparent Km for the substrate Mg•PA possibly by altering its state of aggregation, i.e. lowering of the CMC of the substrate.

DISCUSSION

The localization of PAPase in the microsomal fraction of lactating mammary tissue was consistent with that of other tissues; e.g., 70-90% of PAPase activity of intestine mucosal cells, heart, liver and kidney is associated with the microsomal fraction (21,23,24). Aqueous dispersions of PA is the preferred substrate for microsomal PAPase (21). However, as observed with PAPase from other tissues, the velocity of PA hydrolysis by mammary PAPase increases rapidly when the substrate concentration exceeds the CMC as indicated by the biphasic V/S pattern in Figure 3.

The optimum pH, around 7, is typical of microsomal PAPases (21,25-27), and the rapid decrease above this pH indicates that this phosphatase activity is not due to alkaline phosphatase (28).

The specific activity of microsomal PAPase from mammary tissue (6nm min^{-1} mg^{-1}) was generally higher than the values of one to four (nmoles Pi released per min/mg microsomal protein) observed for tissues of other animals analyzed by similar methods (13,21,26,29). However, we observed that microsomal PAPase from liver of lactating rats was twice as active as the mammary PAPase from the same rats. This may reflect the active involvement of the liver in lipoprotein synthesis and secretion. These, especially the lipids associated with the

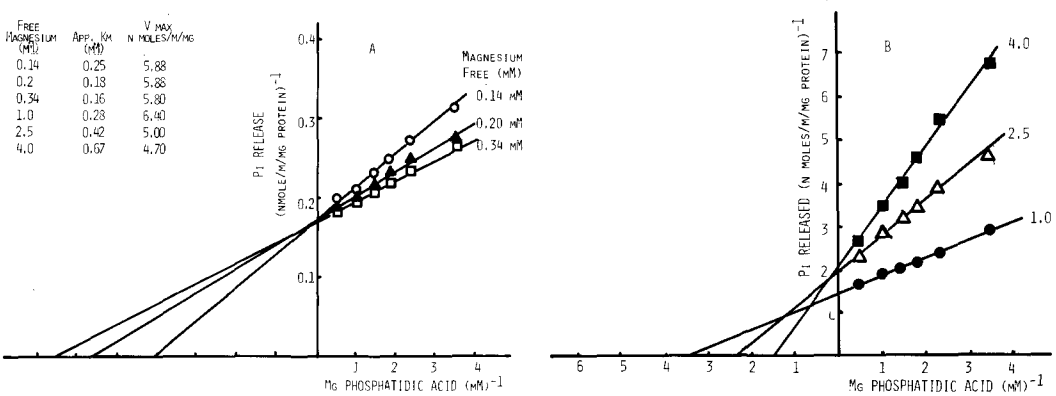

FIG. 5, A,B. The effects of increasing concentrations of magnesium on the kinetic characteristics of phosphatidic acid phosphohydrolase of rat mammary tissue. Free magnesium indicates non-PA bound magnesium. Assay conditions as described in methods.

low density lipoproteins, are used as precursors of milk lipids (30).

The active mammary PAPase may reflect the relatively high rate of glycerolipid synthesis in mammary tissue which in the case of the rat amounts to ca. 3 g/24 hr (3,31). There is some evidence tha PA-phosphohydrolase may be a rate-limiting enzyme in synthesis of triglycerides in liver and adipose (11,13,32). However, there are no data available for mammary tissue on this point. Some data on the relative activities of the other microsomal enzymes involved in TG synthesis in mammary tissue are available. The maximum observed rates for mammary microsomal palmityl-CoA: *sn*-glycerol-3-phosphate acyl transferase; palmityl-CoA:monoacyl-*sn*-glycerol-3-phosphate acyl transferase and dimyristyl-*sn*-glycerol acyl transferase in vitro are (approximately) 2-9, 25-30 and 10-30 nmoles acyl-CoA acyl acylated per mg microsomal protein/min, respectively (3,7,8,16). The maximum activity observed for the PAPase in the present study was 6-7 nmoles/mg protein/min. These data are consistant with the latter enzyme being a possible rate-limiting step. However, because of the many variable factors which can influence data obtained in vitro with microsomal enzymes which utilize amphiphilic substrates (20,23), definitive conclusions cannot be made until appropriate in vivo experiments are conducted.

The apparent Km values obtained under the different assay conditions for PA, 160-700 μM, are within the range of 200 to 500 μM obtained for other microsomal PAPase preparations (13,23,25-26).

Magnesium at concentrations up to 2.5 mM progressively stimulated mammary PAPase, while at concentrations above this activity was depressed. Though literature reports on the effects of Mg on PAPase are conflicting, in general, low concentrations of magnesium (1-3 mM) stimulate while inhibition occurs on higher levels (13,29,33-34). Maximum stimulation occurred at free magnesium $(Mg^{2+})_f$ concentrations of 0.4 mM, and activity of the enzyme was progressively reduced at higher concentrations (1-4 mM) of $(Mg^{2+})_f$. Coras and Shapiro (25) reported that purified PAPase from liver microsomes was slightly stimulated by 0.3 mM free Mg; however, the enzyme activity remained constant at high levels (>4 mM) of this cation. Similar behavior was also noted by Jamdar and Fallon (33). Kako and Patterson (29) found that PAPase from different organelles responded differently to magnesium; e.g., magnesium (1 mM) doubled enzyme activity in microsomal preparations from rabbit heart, whereas, in the case of microsomes from hamster heart, it was inhibitory.

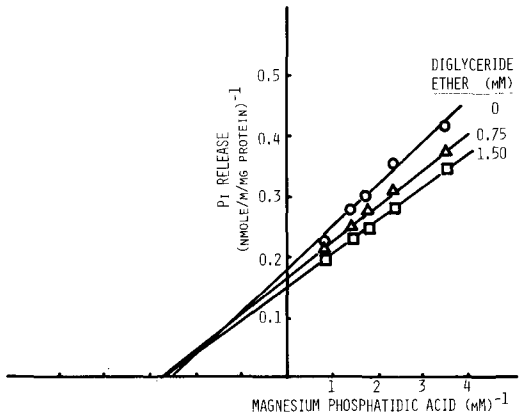

FIG. 6. The effects of dipalmitylglyceryl ether (DGE) on the phosphatidic acid phosphohydrolase activity of rat mammary microsomes.

Jamdar and Fallon (33) claimed that both a magnesium dependent and magnesium independent PAPase exists in adipose tissue. The data for mammary tissue are consistent with this idea because when EDTA was included in assays of the mammary enzyme from which Mg^{2+} was omitted, the specific activity was only slightly depressed. Coras and Shipiro (25) reported that purified rat liver microsomal PAPase did not require Mg^{2+} and, in fact, Mg^{2+} at 1 mM was inhibitory. In our studies, free Mg^{2+} decreased the apparent Km of the enzyme for the substrate from 0.25 to 0.16

TABLE I

The Effect of Inorganic Phosphate on the Rate of Hydrolysis of Phosphatidic Acid by Rat Mammary Microsomes[a]

Concentration of phosphate mM	Glyceride products (nmoles/min/mg protein) A	B
0	6.6	5.3
1	6.6	4.8
2	6.1	4.7
5	4.0	3.7
10	3.2	3.0

[a]H^3-phosphatidic acid (3,000 cpm/ nmole) at a concentration of 1.2 mM was incubated under standard conditions (see Methods) and the radioactivity recovered in the glycerides (1,2-diglycerides, monoglycerides and triglycerides) was measured to indicate the extent of dephosphorylation of PA.

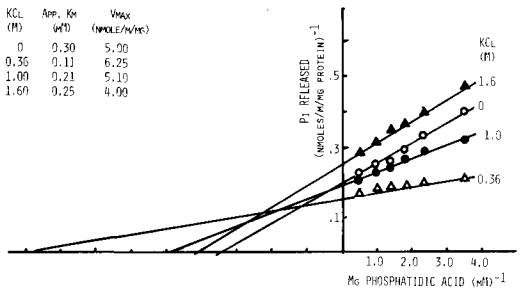

FIG. 7. The effects of potassium chloride on the kinetic properties of phosphatidic acid phosphohydrolase activity of rat mammary microsomes.

mM. Jamdar and Fallon (33) reported that Mg^{2+} at 1 mM decreased Km of the adipose enzyme from 0.55 to 0.24 mM.

The observation that low levels of potassium phosphate and potassium chloride stimulated PAPase may be attributed to the lowering of the critical micellar concentration of PA by these salts. Krag et al. (35) demonstrated that KCl (180 mM) significantly stimulated bacterial PAPase by decreasing Km. In our studies, KCl (0.36 M) reduced the apparent Km from 0.3 to 0.1 mM and concomitantly increased Vmax from 5.0 and 6.2 nmoles Pi released per mg protein per min. While these ions may act by lowering CMC of the substrate, Coras and Shapiro (25) also suggested that monovalent ions caused dissociation of the PAPase from membranes with concomitant increased activity. The increased activity of mammary microsomal PAPase induced by low concentrations of phosphate was in contrast to the behavior of purified PAPase from rat liver which was insensitive to inorganic phosphate (24). This is consistent with the idea that the ions affect the microsomal bound enzyme by causing its dissociation from the membrane.

The absence of any effect following the addition of diglyceride and the slight stimulation of mammary PAPase by diglycerol ether was in contrast to the inhibition by diglyceride of the purified enzyme from rat liver, and the membrane bound enzyme from *Bacillus subtilis* (25,35). The inclusion of phosphatidylcholine in assays of liver microsomal PAPase also stimulated rates of hydrolysis (23). It is possible that these amphiphiles altered the physical state of the PA; e.g., by lowering CMC; by increasing micellar size thereby improving spacing of the substrate PA molecules, or by eluting the enzyme from the membrane.

These data indicated that the properties of microsomal PAPase from rat mammary tissue are generally similar to those of PAPase from other mammalian tissues. Future studies will involve monitoring the activity of this enzyme in mammary tissue during lactogenesis.

ACKNOWLEDGMENT

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Carotene in Bovine Milk Fat Globules: Observations on Origin and High Content in Tissue Mitochondria

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ABSTRACT

The location and origin of carotenoids in bovine milk fat globules was investigated using spectral absorption of lipid solutions at 461 nm to quantitate carotene. Release of membrane from globules as a result of churning to butter or by freezing and thawing of the globules yielded membrane preparations which were devoid of carotene. Globule cores from these procedures exhibited carotene concentrations comparable to those in total milk lipids. Fractionation of lactating bovine tissue and analysis of lipid extracts revealed that the intracellular fat droplets have carotene concentrations approximating those of secreted globules. However, intracellular membranes of the tissue, particularly the mitochondria, are much richer in carotenoids than formative or secreted fat globules. The evidence indicates that bovine milk fat globules acquire carotene during their formation in the cell, but that some minor fraction of the total carotene may be extracted from the enveloping secretory membrane. Mean carotene values (μg/g of lipid) for fractions from three samples of lactating tissue were: whole tissue 47, mitochondria 461, microsomes 69, cytosol 67, fat droplets 8, milk 9. One tissue analysis indicated that Golgi membranes contain somewhat more carotene than do microsomes.

INTRODUCTION

From 75 to 95% of the carotenoids of bovine milk are β-carotene. It is an important form (provitamin) of vitamin A in milk and milk products and is responsible for the (natural) yellow color of butter. The amount of carotene in milk is influenced by a number of factors, the most important of which is dietary intake. Within a week or so of being placed on pasture in the spring, cows produce milks with sharply increased carotene contents. It is notable that the milk fat of many species of ruminants is colorless, e.g., that of the goat. Factors influencing the vitamin A and carotene content of bovine milk have been reviewed (1).

Despite extensive biochemical investigations of the milk lipids in the past several decades, the location of carotene in the milk fat globule remains somewhat confused. White et al. (2) observed that carotene distribution in milk fat globules is related to globule size with small globules containing more carotene than large ones. This led them to suggest a surface disposition for the pigment. Thompson et al. (3) reported high levels of carotene in milk fat globule membrane (MFGM) lipids, although the membrane as such was not isolated from their buttermilk preparations. Patton and Fowkes (4) noted that the floating lipid fraction from homogenates of lactating bovine tissue were less pigmented (yellow) than milk lipids obtained from the animal prior to slaughter. They suggested that the milk fat droplet acquires its carotene when enveloped by plasma membrane at secretion. Keenan et al. (5) reported observable levels of carotene in plasma membrane preparations from lactating bovine tissue. However, such membrane would not be derived exclusively from lactating cells or yet more precisely, the apical (secretory) membrane of such cells. In distinction to the latter membrane, the basal membrane, which transports materials into the cell, might well contain carotene. Smith et al. (6) have observed that membrane removed from bovine milk fat globules by a detergent extraction process contained essentially no carotene. This is consistent with our own recent observations of MFGM prepared by other methods. Thus, we undertook further investigation of the matter. MFGM has been the subject of review (7,8).

MATERIALS AND METHODS

All samples of milk and lactating tissue were obtained from individual pastured Holstein cows. The four milks used to prepare MFGM were gathered in July and August, each from a different animal. The tissues and their corresponding milk samples were secured from four additional animals in July, September and November. Carotenoid content of milk fat drops rapidly in our locale when cows are shifted to stored feeds, usually in late November or early December.

Preparation of Milk Fat Globule Membrane (MFGM)

Two classical procedures were used to prepare MFGM from fresh morning milk. One was by churning (9) and the other by freezing and thawing (10) of washed milk fat globules.

In the former procedure, approximately 10 liters of very fresh milk was separated at 37 C into cream and skim milk with the aid of a laboratory scale cream separator. The cream (25% fat) was washed four times by dispersion in 3 to 4 volumes of 0.25 M sucrose containing 0.002 M $MgCl_2$ and repassage through the separator. The washed cream was cooled to 12 C and churned in a small Hobart-type mixer. In this process, the membrane is released from the fat globules which then adhere to each other to form butter. The buttermilk containing the membrane material was centrifuged at 49,000 x g for 2 hr to yield pellets of MFGM. In the freeze-thaw procedure, portions of the washed cream were placed in centrifuge tubes and spun at 10,000 x g for 20 min in the cold (4 C). These tubes were placed in a freezing cabinet (-4 C) for 24 hr, then warmed slowly to 45 C and centrifuged at 49,000 x g for 2 hr to sediment the released membrane. Portions of the original milks and various fractions produced in the preparations of MFGM; i.e., washed cream, washed cream buttermilk, butter, etc., also were saved for analyses.

For the purpose of comparing the carotenoid content of MFGM with that of the bulk fat phase of the globules from which the membrane was derived, butteroils from the churning and freeze-thaw procedures were obtained. For the latter, this simply involved removal of the oil layer at the time the membrane was sedimented. For the former, 20 g of butter was melted at 45 C in a 50 ml centrifuge tube and centrifuged at 10,000 x g and ambient temperature for 40 min. The clear oil layer was used for analyses. Butteroil was also prepared from commercial (uncolored) butter by this procedure.

Fractionation of Tissue

Prior to slaughter of the animals, 100 ml samples of milk were collected to enable comparisons of carotene contents in milks and tissues. Fifty g of the fresh lactating tissue was cut into 0.5 cm fragments, which were washed in cold tap water and placed in a Waring Blender containing 300 ml of ice cold 0.25 M sucrose solution. The mixture was homogenized at maximum power for 1 min. Various fractions were derived by centrifuging this homogenate in 50 ml tubes at 4 C in a Sorvall Model RC-5B centrifuge as follows. An initial centrifuging at 1000 x g for 12 min yielded three fractions: a cell cream on top, a sediment (debris), and an intermediate layer. The cell cream and debris were isolated and retained for analysis and the middle layer was transferred to fresh tubes and centrifuged at 10,000 x g for 20 min. A slight fatty layer from this spin was removed and added to the first cell cream. The sedimented pellets were suspended in a few ml of sucrose and pooled as a crude mitochondrial fraction and the supernatant was placed in fresh tubes and centrifuged at 49,000 x g for 2 hr. This latter yielded a microsomal fraction (pellet) and a supernatant which was designated cytosol. These fractions including some of the original homogenate were submitted to lipid extraction and analysis as described following. The July sample of tissue was used to isolate Golgi membrane and rough endoplasmic reticulum according to the method of Keenan et al. (11). For purposes of characterization, fractions were fixed and viewed with an electron microscope using procedures previously described (11).

Lipid Extraction and Analysis

Milk, milk components derived in the isolation of MFGM, and the various tissue fractions were extracted to obtain lipids by the Roese-Gottlieb procedure (12). Lipids in fractions from the July tissue (only) were extracted by the Folch procedure (13). For quantitation, lipids were dried to constant weight under vacuum (15-20 mm Hg and 38 C). Spectral properties of lipids in the visible region (350 to 600 nm) were determined in 19:1 (v/v) chloroform/methanol with a Beckman Model 25 spectrophotometer. Some spectra were obtained with a Bausch and Lomb Spectronic 20 instrument. Carotenoids in the milk and tissue extracts were analyzed as described by Davis (14) using E 1% cm of 2396 (chloroform). Both authentic β-carotene (Hoffmann-LaRoche Inc., Nutley, NJ) and the various lipids of this study exhibited an absorption maximum of 461 nm. It was used as the setting for the carotene analyses. Efforts were made to protect samples from light and to complete carotenoid analyses quickly. However, amber glassware was not employed.

Lipid phosphorus was determined by the method of Rouser et al. (15). The results were multiplied by 25 to convert them to the quanties of phospholipid. Cholesterol was assayed by the procedure of Bachman et al. (16).

RESULTS

While there is good evidence that carotenoids are the normal pigments in bovine milk fat (1), we wished to check this fact in terms of our own samples and spectroanalytic methods. In Figure 1, absorption spectra in the visible region are presented for authentic β-carotene and butteroil prepared from a sample of com-

mercial uncolored butter. Both show the characteristic maximum at 460 to 465 nm and inflections in the regions of 493, 438 and 408 nm. Whenever yellow color was observable in the various milk, fat globule and tissue lipid fractions of this study, these same absorption properties were seen with the spectrophotometer. On silicic acid column chromatography (17) of milk and mammary tissue lipids, the only pigmented band we have ever seen is that corresponding to carotene in the hydrocarbon fraction. Thus, absorption of 461 nm was used to quantitate carotenoids in the lipid fractions.

The following yield data from one of the four preparations of MFGM by the churning procedure are representative: passage of 8.6 kg of fresh milk through the separator gave 6.6 kg of skim milk and 2.1 kg of cream. Following washing and reseparation (4 times), this cream yielded 250 g of butter and 1100 ml of buttermilk. Centrifugation of the washed cream buttermilk deposited 3.8 g of wet MFGM. Roese-Gottleib extraction (12) of the membrane suspended in 10 ml of 0.25 M sucrose solution produced .2937 g of total lipid. The phospholipid and cholesterol contents of this lipid were 41.0% and 4.1%, respectively. These latter values are in the normal range for this membrane. Glycerides generally make up from 53 to 74% of the lipids (7). In other studies, we have shown that MFGM made by these procedures has characteristic membrane bilayer ultrastructure (18) and marker enzymatic activities (5'nucleotidase, nucleotide pyrophosphatase, Mg^{2+} ATPase) for plasma membrane (19). On the basis of lipid phosphorus in the washed cream compared to that in the membrane lipids, the yields of membrane for three of the trials averaged 19%. In one of the trials, it was found that yield of membrane from the washed cream buttermilk was 62%.

The lipid extracts of the four membrane preparations showed no visible evidence (yellow color) of carotenoids. A slight brownishness could be seen which in our experience is characteristic of concentrated solutions of phospholipids. The absence of carotenoid in all the MFGM preparations was confirmed spectrophotometrically. As shown in Figure 2, membrane lipids exhibited no absorption maximum for carotenoids (460-465 nm), whereas both the whole milk lipids and butteroil refined from the butter were bright yellow and showed this absorption. The somewhat lower level of carotenoid in the butteroil as compared to the milk lipids from which it was derived may be due to (oxidative) losses during the separation, washing, churning and rendering of the oil.

FIG. 1. Absorption spectra for butteroil from commercial butter against hexane (upper) and β-carotene in chloroform (lower).

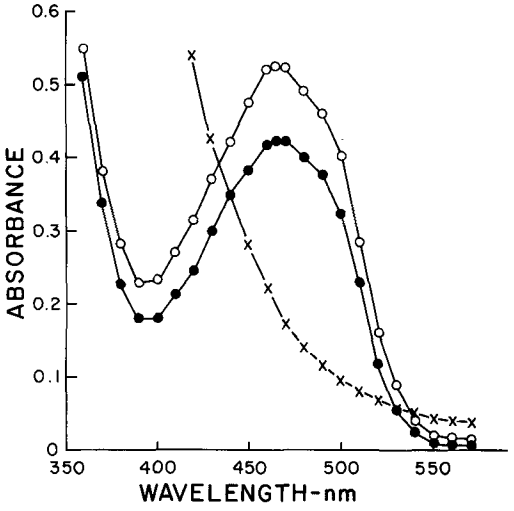

FIG. 2. Absorption spectra of lipid extracts in the preparation of milk fat globule membrane: o——o whole milk lipids; •——• butteroil from that milk; X——X milk fat globule membrane lipids from that milk. 1.0 g made to 3.5 ml with chlroform/methanol, 19:1, for the milk lipids and butteroil, 294 mg of the membrane lipid similarly made to 3.5 ml.

When a batch of washed cream was split and some of it used to produce MFGM by the freeze-thaw procedure and the rest to produce the membrane by churning, neither preparation yielded lipid extracts with observable carotenoids. The extracts were relatively colorless and their spectra, Figure 3, were sloping curves closely resembling that for MFGM in Figure 2. Spectra for total lipids of washed cream, butteroil rendered from the butter and oil released in isolating membrane by the freeze-thaw procedure were nearly superimposable, Figure 4. As in Figure 2, solvent extraction gave slightly better recovery of carotenoid than did rendering oil from butter.

Since there appeared to be no carotene in the MFGM, it was felt that distribution of the pigment in lactating tissue should be examined, particularly the amount in a fraction representing milk fat globules in the presecretory state. The carotene concentrations in lipids from this fraction (cell cream), from other tissue fractions, and corresponding milks of three cows are given in Table I. The cell cream lipids from tissues of the three animals contained similar concentrations of carotene to those found in the corresponding milks. The data also reveal that lipids from all of the other tissue fractions had higher concentrations of carotenoids than did the milk lipids. The particularly high levels in the mitochondrial fraction were further investigated. The crude fraction (tissue B) was judged by electron microscopy to be 50% mitochondria with rough microsomal vesicles as the principal contaminant. Washing and resedimenting the crude preparation at 10,000 x g increased the carotene conccentration from 466 to 974 μg/g of lipid. This exhibited the characteristic absorption spectrum (λ max 461 nm) for β-carotene. The debris and tissue homogenate values for carotene in Table I are similar since the debris would contain unbroken pieces of tissue. It is suspected that these values are low in tissue A because of neutral fat accumulation in the udder as the animal was going dry.

One explanation of the absence of carotene in MFGM concerns its possible exclusion as a secretory membrane component at the level of the Golgi apparatus. Thereby, Golgi membranes might have lower concentrations of carotene than endoplasmic reticulum, and secretory vesicles might exhibit still lower concentrations than Golgi membrane. We examined this question with an additional sample of tissue (July). The values for rough endoplasmic reticulum, Golgi and mitochondrial fractions were 100, 159 and 322 μg of carotene/g of lipid. Thus, it does not appear that the Golgi apparatus is depleted of carotene or is intermediate in value between endoplasmic reticulum and secretory membrane.

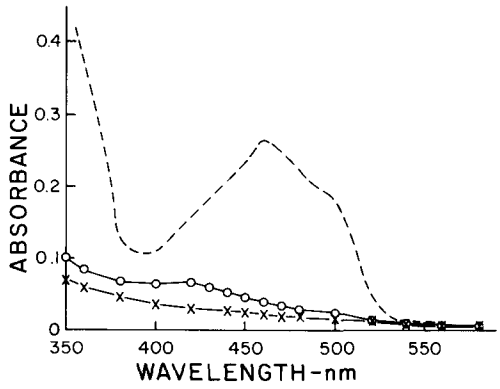

FIG. 3. Absorption spectra of lipid extracts in the preparation of milk fat globule membrane: o——o lipids from membrane prepared by churning washed cream (65 mg of lipid in 3.5 ml of 19:1 chloroform/methanol); X——X lipids from membrane prepared by freezing and thawing the same washed cream (20 mg of lipid in 3.5 ml of 19:1 chloroform/methanol); ---- spectrum of butteroil released in the freezing-thawing preparation of membrane.

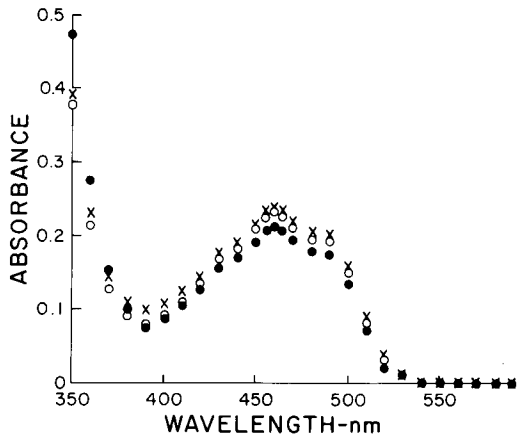

FIG. 4. Absorption spectra of lipid extracts in the preparation of milk fat globule membrane: X——X washed cream lipids; o——o freeze-thaw butteroil from the same washed cream; •——• butteroil from the same washed cream. 1.0 g of each lipid made to 3.5 ml with 19:1 chloroform-methanol.

DISCUSSION

It is most likely that the carotene of milk fat globules reaches the mammary gland by way of the circulation. All three of the principal serum lipoproteins (very low density, low density and high density) of lactating bovine blood carry

carotenoids (B.C. Raphael and S. Patton, unpublished). Moreover, lipids of bovine serum lipoproteins are principal precursors of milk triglycerides (20,21), and in the lactating rat it has been shown that all three of the foregoing serum lipoproteins can transfer cholesterol to milk (22). However, the molecular events in movement of carotenoids into the mammary cell are not known.

The data of Table I indicate that carotene is associated with the various endomembranes of the lactating cell. Formation of the milk fat droplet appears to be initiated in the matrix of the endoplasmic reticulum at the base of the cell (for review, see ref. 23 and 24). This location should be rich in metabolites entering the cell from the circulation. It may be there that the bulk of the carotene is transferred to forming fat droplets. This would explain the observation of White et al. (2) that smaller milk fat globules have more carotenoids per weight of fat than larger ones. Larger globules may undergo greater growth in a more dilute carotene environment in the apex of the cell. Peixoto de Menezes and Pinto da Silva (25) have proposed from freeze fracture studies of forming milk fat droplets that droplet growth occurs by transfer of membrane lipids at the droplet surface. This would be a plausible mechanism to explain uptake of carotene from endomembranes by the droplet.

Our findings are not consistent with the hypothesis that carotene of milk fat globules is acquired from apical plasma membrane at secretion. Examination of MFGM prepared by two methods, churning and freeze-thaw, revealed it to be devoid of carotene. Preparation of MFGM by yet another method, detergent extraction (6), also yielded membrane free of carotenoids. Rather, as in our study, the core lipids of the globule accounted for the pigment.

It may be reasoned that carotene is promptly extracted from the plasma membrane into the fat droplet at secretion, thus accounting for the virtual absence of carotene concentration in MFGM. Assuming a globule of 3 μm diameter bound by a membrane 100 Å thick, the volume of the membrane can be calculated using the formula for volume of a sphere $V = 4/3\ \pi\ r^3$. The membrane volume is approximately one-fiftieth of the globule volume, which indicates that it would have to contain 50 times the carotene concentration found in milk fat to supply all of the carotene for the latter. This concentration in the membrane would be 300 to 1000 μg/g lipid based on the data of Table I. While it seems reasonable that the plasma membrane would contain some carotene, it does not seem to be a plausible source of all the globule carotene. The fact that the cell cream (intracellular fat droplets) fraction, Table I, has virtually the same carotene concentration as secreted globules also refutes such a source. Analysis of secretory vesicle membranes for carotene content should shed further light on this matter since they appear to be the immediate precursor of apical plasma membrane. Two additional possibilities regarding the lack of carotene in MFGM are destruction by oxidases or selective removal at the time of fat droplet secretion. Results from a recent freeze fracture study (26) of membrane events at secretion may be interpreted to show that some plasma membrane components are selectively retained by the cell.

TABLE I

Distribution of Carotenoids in Mammary Tissue Fractions and Milks from Three Holstein Cows[a]

Fraction	A (9/27)	B (11/7)	C (11/30)
	μg carotene/g of lipid		
Mitochondria	799	466	117
Microsomes	162	17	27
Cytosol	127	51	22
Debris	39	76	50
Whole tissue	31	60	50
Cell cream	10	10	4
Milk	10	11	6

[a]These were three Holsteins slaughtered at the dates indicated. Cow A was nearly dry. B and C were giving about 14 kg of milk per day.

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The Effect of Oral Contraceptives on Mononuclear Cell Cholesteryl Ester Hydrolase Activity

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ABSTRACT

The influence of sex steroids on mononuclear cell cholesteryl ester hydrolase (CEH) activity in premenopausal women and women on combined estrogen-progestin oral contraceptives has been studied. In addition, plasma and mononuclear cell cholesterol and esters were measured along with plasma estrogen and progesterone levels. Mononuclear cell CEH activity in control women is highest on Day 20 of their menstrual cycle. The control women had significantly higher CEH activities than women on oral contraceptives. Plasma esters were higher in the oral contraceptive group. However, in mononuclear cells, free cholesterol but not cholesteryl esters were higher in women on oral contraceptives.

INTRODUCTION

Atherosclerosis remains the most prominent disease numerically in the United States, since it accounts for the first and third causes of death and disability among Americans due to coronary heart disease and strokes. One of the features of atherosclerosis is the accumulation and deposition of cholesterol and its esters in the arterial wall. This accumulation may, in part, be due to a deficiency of lysosomal cholesteryl ester hydrolase (CEH) (1-4). Two human diseases, namely, Wolman's and Cholesteryl Ester Storage Disease (CESD), are characterized by pronounced tissue accumulation of cholesteryl esters and by profoundly depressed CEH activity (5-7). In Wolman's disease, Cortner and coworkers have shown the complete absence of a lysosomal acid lipase in cultured fibroblasts (6) and in mononuclear cells (7). Data from our laboratory have shown that individuals with symptomatic atherosclerosis have significantly lower mononuclear cell CEH levels than those who are asymptomatic (unpublished observations). These studies suggest a role of reduced CEH in atherogenesis. However, the factors that modulate CEH activity in atherosclerosis are not known. One of the factors that may play a role in atherogenesis is hormones, since it has been demonstrated that women taking oral contraceptives can develop accelerated atherosclerosis and have an increased incidence of coronary heart disease (8-10). The clinical recognition of atherosclerosis in women before menopause is uncommon and rare in the absence of hypertension, diabetes, anemia, or myxedema. If CEH and plasma lipids are involved in the pathogenesis of the disease and, as women are relatively immune before menopause, further information obtained by studies of mononuclear cell CEH and plasma lipids during the normal menstrual cycle and in women on oral contraceptives is of considerable clinical importance.

PROCEDURES

Subjects

Premenopausal women were selected from the staff of the Medical Center to form an approximately homogeneous group with respect to height, weight and age. The mean age was 26 (range: 19-30 years); the mean height was 5'5" (range: 5'1"-5'9"); and none of the women was overweight. All subjects were healthy according to their history. Subjects with hyperlipidemia or abnormal routine laboratory tests of renal and hepatic functions were excluded from the study. None of the women was receiving any form of medical or vitamin therapy. Only those women taking oral contraceptives for contraception were studied, as opposed to those regulating their menstrual cycles. Nine of the ten women on oral contraceptives were receiving Ortho Novum 1/50; the tenth person was receiving Norlestrin 1/50.

Collection of Blood Samples

Samples of venous blood (20 ml) were taken from each unfasted subject in a uniform manner. All samples were obtained between 8:30 a.m. and 9:30 a.m. Samples of venous blood were taken at days 5, 10, 15, 20 and 25 of control women's menstrual cycle. The day of the cycle in each woman was determined from day 1 of menses. Women on oral contraceptives were sampled on days 5, 10, 15 and 20 after their last pill. The samples were assayed immediately for mononuclear cell CEH activity and aliquots of plasma and mononuclear cells were processed for cholesterol and cholesteryl ester determinations. Aliquots of plasma were quick frozen for hormone measurements by radioimmunoassay.

Biochemical Analysis

The acid CEH activity of the mononuclear cells was measured as previously reported (11) but will be described in brief. Optimum assay conditions for mononuclear cell CEH were determined for pH (4.5) and substrate concentration (3-4 nmoles). Hydrolysis was linear with incubation time (up to 90 min) and with protein concentration (40-120 μg/incubation). Each 10 ml of blood was diluted with 30 ml normal saline and carefully layered upon 10 ml Lymphoprep (sodium metrizoate/Ficoll). The samples were then centrifuged for 30 min at 20 C, at 400 x g (measured at the interface between blood and Lymphoprep). The middle band, containing the mononuclear cells, was removed and centrifuged again to pellet the cells. The mononuclear cells were then washed several times with saline. After the final wash, the cell suspension was transferred to a 2.5 ml Dounce homogenizer. The cell suspensions were uniformly homogenized in saline 30 times prior to adding them to the CEH assay system. Disruption of the cells by more vigorous means failed to show any increased CEH activity in this assay system. Routinely 1-1.5 x 10^6 cells are added to the incubation medium. The assay medium contains 1.3 ml 0.2 M acetate buffer, pH 4.5, 0.1-0.2 ml mononuclear cells and 3-4 nmoles ^{14}C-1-cholesteryl oleate (SA 55 mCi/mmole, New England Nuclear) injected in 20 μl acetone. In control samples, p-chloromercuriphenyl sulfonic acid (PCMPS) was added to inhibit the enzyme. At 3.3 x 10^{-3} M concentration the PCMPS completely inhibited the CEH activity. After incubation of the samples for 1 hr at 37 C, the samples were extracted with 3.0 ml benzene/chloroform/methanol, 1:0.5:1.2, 0.6 ml of 0.3 M NaOH, was then added, stirred and centrifuged. A 0.5 ml aliquot of the upper phase (containing free oleic acid) was added to a toluene-10% Beckman Biosolv scintillation cocktail and the radioactivity determined.

Plasma and mononuclear cell cholesterol and ester levels were measured in each subject. A 0.100 ml aliquot of plasma and a 0.250 ml aliquot of the mononuclear cell preparation was extracted 2x with 5 ml $CHCL_3$/MeOH, 2:1. The samples were then evaporated to dryness, derivatized with TMS-Sil Prep (Applied Science Laboratories) and assayed on a 5830-A Hewlett-Packard gas chromatograph (3% SE-30 on 10% Gas Chrom, Applied Science Laboratories) to obtain free cholesterol levels. The samples were then saponified, extracted and again derivitized to obtain the total cholesterol concentration. The samples were corrected for procedural losses by including ^{14}C-cholesterol and ^{14}C-4-cholesteryl oleate standards in each assay. The recoveries for each compound were greater than 90% (range: 90-99%). The values for cholesterol and its ester are 10-15% lower than standard clinical colorometric methods. Protein concentration in each mononuclear cell sample was determined by the method of Lowry et al. (12). The statistical significance of mean differences was determined using Student's t-test.

Plasma levels of estrogens and progesterone were determined for control women at each test period by radioimmunoassay. The assays were performed at the Oregon Regional Primate Research Center. The procedures, specificity, precision and accuracy of the method are well documented (13,14). The estrogen values represent the sum of estradiol and estrone. At each test period, correlation coefficients between progesterone and CEH and estrogens and CEH were calculated to disclose a possible relationship between sex steroid level and CEH activity.

The lysosomal marker enzymes β-glucuronidase and β-D-N-acetylglucosaminidase were assayed in the mononuclear cells of control women during each test period as previously described (15,16).

RESULTS

The Effect of Oral Contraceptives on CEH (Table I)

The mononuclear cell CEH levels in control and in women or oral contraceptives are shown in Table I. Normally menstruating women showed a significant rise in CEH levels on day 20 of the menstrual cycle, $p<0.05$. The enzyme activity in women on oral contraceptives was significantly lower than control women in three of four test periods.

TABLE I

The Effect of Oral Contraceptives on Mononuclear Cell CEH Activity[a]

Day of cycle	Control (C)	Oral contraceptive (OC)	P
5	1383 ± 257	842 ± 168	<.05
10	1570 ± 222	896 ± 108	<.01
15	1318 ± 225	1083 ± 237	NS
20	1950 ± 202	958 ± 179	<.005
25	1530 ± 352	---	---

[a]Values are expressed as pmoles/mg P/h, Mean ± SE; n = 9 controls and 10 oral contraceptive.

Plasma Levels of Cholesterol and Esters (Table II)

The *free* cholesterol levels in normal cycling

women and in the oral contraceptive group did not vary significantly during the cycle nor between the two groups. However, the women on oral contraceptives had significantly higher *ester* levels than the control women in three of the test periods.

TABLE II

The Effect of Oral Contraceptives on Plasma Cholesterol and Ester Levels (mg/100 ml)[a]

Day of cycle	Control (C)	Oral contraceptive (OC)	P
	Free Cholesterol		
5	47 ± 2	52 ± 3	NS
10	49 ± 3	47 ± 3	NS
15	49 ± 2	48 ± 2	NS
20	41 ± 2	47 ± 3	NS
	Cholesteryl esters		
5	86 ± 8	113 ± 12	<.05
10	95 ± 13	104 ± 11	NS
15	76 ± 4	117 ± 13	<.005
20	77 ± 4	133 ± 17	<.005
	Cholesterol/ester ratio (paired data)		
5	0.53 ± .04	0.49 ± .06	NS
10	0.58 ± .05	0.49 ± .03	<.05
15	0.60 ± .05	0.43 ± .04	<.01
20	0.54 ± .03	0.36 ± .03	<.005

[a]Values are Mean ± SE; n = 9 controls and 10 oral contraceptive.

Mononuclear Cell Cholesterol and Esters (Table III)

Cholesterol levels in women on oral contraceptives were significantly higher in each test period than control women. However, the esters were not significantly different in three of the four test periods. On day 5 control women had higher ester levels than the oral contraceptive group.

Ratios of Cholesterol to Cholesteryl Esters

When paired ratios of *plasma* cholesterol to esterified cholesterol were compared between control and oral contraceptive groups, the ratio of free/esterified was significantly higher in the control group in three of four test periods (Table II). In *mononuclear* cells, on the other hand, the cholesterol/cholesteryl ester ratio was significantly *lower* in the control group during the four test periods (Table III).

Estrogen and Progesterone Concentrations in Plasma (Table IV)

Values for plasma concentrations of estrogens and progesterone fall in the expected range for normal premenopausal women. Due to the fact that blood samples were only obtained every five days, it was not possible to detect the midcycle estrogen peak in all cases; however, the elevated progesterone concentration characterizing the luteal phase was present in all cycles. There was no significant correlation between plasma estrogen levels and CEH activity during any of the five sample periods. In the case of progesterone vs. CEH, a positive correlation of 0.80 ($p < 0.01$) exists on day 5 of the cycle and a negative one of -0.81 on day 25 ($p < 0.01$).

Lysosomal Marker Enzymes (Table V)

Mononuclear cell lysosomal enzymes β-glucuronidase and *N*-acetylglucosaminidase did not vary significantly with stages of the menstrual cycle in normal premenopausal women.

DISCUSSION

It has been proposed that female sex hormones protect premenopausal women from developing coronary heart disease (17-19). Hormones, particularly estrogens, exert multiple metabolic effects and, at present, it is not possible to determine which ones are principally responsible for the protective effect(s). Sex steroid hormones appear to have a major

TABLE III

The Effect of Oral Contraceptives on Mononuclear Cell Cholesterol and Ester Levels (μg/mg P)[a]

Day of cycle	Control (C)	Oral contraceptive (OC)	P
	Free Cholesterol		
5	15.9 ± 4.0	27.2 ± 2.5	<.025
10	17.2 ± 3.2	29.7 ± 2.7	<.005
15	14.9 ± 2.8	29.4 ± 2.7	<.005
20	14.1 ± 2.6	33.0 ± 3.6	<.005
	Esters		
5	9.3 ± 1.9	4.4 ± 1.0	<.0.25
10	9.8 ± 2.0	12.0 ± 4.4	NS
15	11.2 ± 3.1	9.7 ± 2.1	NS
20	14.8 ± 3.1	9.0 ± 2.6	<.10
	Cholesterol/ester ratio (paired data)		
5	2.3 ± 0.65	13.9 ± 4.4	<.01
10	1.8 ± 0.40	6.3 ± 1.9	<.025
15	2.8 ± 1.10	5.2 ± 1.3	<.10
20	1.5 ± 0.50	5.3 ± 1.3	<.01

[a]Values are Mean ± SE; n = 9 controls and 10 oral contraceptive.

TABLE IV

Plasma Progesterone and Estrogen Levels in Control Women[a]

Hormone	Day 5	Day 10	Day 15	Day 20	Day 25
Progesterone	0.91 ± 0.04	0.95 ± 0.14	3.50 ± 1.69	14.50 ± 2.17	8.79 ± 1.81
Estrogens	83.5 ± 4.1	164.9 ± 20.2	251.8 ± 42.4	189.5 ± 27.4	141.8 ± 22.8

[a]Values are Mean ± SE; progesterone values are nanograms/ml; estrogen values are picograms/ml; N = 9 controls.

TABLE V

Mononuclear Lysosomal Hydrolases

Enzyme[b]	Day 5	Day 10	Day 15	Day 20	Day 25
Beta-D-glucuronidase	554 ± 33	683 ± 85	661 ± 89	590 ± 48	665 ± 63
Beta-D-N-acetyl-glucosaminidase:					
Total	2571 ± 253	2887 ± 396	2606 ± 257	2488 ± 390	2628 ± 255
Heat stable	1162 ± 110	1306 ± 196	1137 ± 131	1109 ± 82	1180 ± 144
Heat labile[c]	1409 ± 146	1581 ± 203	1468 ± 138	1380 ± 83	1448 ± 122

[a]Values are Mean ± SE.

[b] Units are nanomoles of artificial substrate hydrolyzed/mg protein/hr at 37 C.

[c]Heat labile of total activity.

influence on serum lipoprotein distribution. For example, premenopausal women have lower concentrations of very low density lipoproteins and higher concentrations of HDL2 than men in similar age-matched groups (20).

The widespread use of oral contraceptives during the last 15 years has shown that *exogenous* sex steroids and their analogs can also cause substantial changes in serum lipoprotein levels (21). Studies of women on oral contraceptives have thus provided material to examine several parameters associated with atherosclerosis. Triglyceride concentrations increase, along with low levels of very low density lipoproteins (22-24). Concentrations of cholesterol and low density and high density lipoproteins may also increase, but the changes are variable and appear to depend on the relative amounts of estrogen and progestin in the preparation (8). Recent evidence has shown for the first time that estrogen also significantly inhibits cholesterol synthetase activity and stimulates cholesteryl ester hydrolysis (25).

Based on these findings, we began investigations on the influence of sex steroids on cholesterol metabolism in mononuclear cells in premenopausal women and women on combined estrogen-progestin oral contraceptives. In our studies of 9 premenopausal women, the levels of mononuclear cell CEH were significantly higher on day 20 than on day 5 or day 15 of their menstrual cycles. In fact, 6 of 8 women showed a 50.7 ± 10.7% (mean ± SE) rise in CEH activity on day 20 compared to day 15. However, the 10 women on oral contraceptives did not show any significant fluctuations during the similar test periods. More important, the oral contraceptive group had significantly *lower* mononuclear CEH levels than the control women (Table I). In addition, the levels of the lysosomal marker enzymes β-glucuronidase and N-acetyl β-D-glucosaminidase in controls did not vary significantly during the test periods (Table V).

In each subject, plasma cholesterol and cholesteryl ester levels were measured. The plasma cholesterol levels did not significantly change *during* the test period in any group (Table II). Also, the plasma cholesterol levels did not vary significantly *between* the groups. Although the plasma cholesteryl ester levels did not significantly fluctuate in each group *during* the test period, the levels *between* the groups were significantly different (Table II).

In three of four test periods, the plasma cholesteryl ester levels were significantly lower in control women than women on oral contraceptives. When paired cholesteryl/cholesteryl ester ratio determinations were made, the differences between the groups were significant (Table II). In three of four test periods, the control women have significantly higher plasma cholesterol/cholesteryl ester ratio than the oral contraceptive group. The elevated ratio reflects

lower levels of ester in the control group. Although lecithin/cholesteryl acyl transferase (LCAT) reaction in plasma is thought to be the main source of cholesteryl esters in plasma, recent evidence has failed to show a sex difference in this enzyme (26,27) nor between normally cycling women and those on oral contraceptives. They did, however, find the molar LCAT rate in women significantly lower on day 10 than on day 23 of the menstrual cycle. Other factors in addition to LCAT may also be acting to cause elevated plasma ester levels in the oral contraceptive group.

In addition, in each subject we measured mononuclear cell cholesterol and ester levels. The mononuclear cell cholesterol levels did not significantly vary *during* the test periods in each group. However, the levels of cholesterol *between* the two groups was significantly different (Table III). In each test period, the level of cholesterol was higher in the oral contraceptive subjects. The ester levels did not vary significantly in three of four test periods between the two groups. The cholesterol/cholesteryl ester ratio was significantly higher in women taking oral contraceptives; the elevated ratio reflects the higher *free* cholesterol levels in the oral contraceptive group (Table III). It is interesting to note the higher cholesterol/cholesteryl ester ratios present in mononuclear cells than in plasma.

Our preliminary studies of ten women on oral contraceptives, showing significantly lower mononuclear CEH levels than control premenopausal women, are also supported by earlier studies. Since the hypophyseal system in the oral contraceptive women was suppressed, the lower CEH levels observed suggests that CEH activity is modulated by hypophyseal hormones. It has been shown that hypophysectomy reduces the specific activity of CEH in steroidogenic tissues (28) and results in a significant accumulation of tissue cholesteryl esters (29,30). Furthermore, the rates of both cholesterol esterification and hydrolysis are increased in early pregnancy and these changes may be mediated by pituitary and chorionic gonadotropins (31-33). In addition, luteinizing hormone induces depletion of cholesteryl esters in steroidogenic tissues (34,35). Prolactin or ACTH can also maintain CEH activity comparable to that in animals with an intact pituitary, indicating action similar to the action of luteinizing hormone (28). Recent data indicate that corticotrophin activates adrenal esterase by a cAMP-dependent protein kinase (36). These findings in experimental animals and humans indicate an association between CEH and female sex steroids.

The demonstration of profoundly depressed CEH levels in Wolman's and Cholesteryl Ester Storage diseases (5-7), the latter with demonstrated atherosclerosis, and our own data that individuals with symptomatic atherosclerosis have significantly lower mononuclear cell CEH activity (unpublished observations) strengthen the theory which postulates a role of depressed CEH levels in atherosclerosis (1-4). We found that women taking oral contraceptives, known to have an increased incidence of early atherosclerosis (8-10), display changes in cholesterol and its ester in plasma and in mononuclear cells with reductions in their mononuclear cell CEH activity. These findings suggest an association between exogenous sex steroids or reduced endogenous sex steroids and CEH activity.

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Identification of Lipoxygenase-Linoleate Decomposition Products by Direct Gas Chromatography-Mass Spectrometry

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ABSTRACT

Lipoxygenase, prepared from Virginia-type peanuts, was used to catalyze the oxidation of linoleic acid and methyl linoleate to form the C-9 and C-13 hydroperoxides. These reactions were monitored by rapid unconventional direct gas chromatography-mass spectroscopy. An aliquot of the enzymatic reaction mixture, without prior extraction or chemical modification, was secured directly into the heated (40-70 C) or nonheated (room temperature) injection system. When the reaction mixture was analyzed at room temperature, only hexanal was found. At elevated temperatures, five major and several minor components were identified. The predominant compounds identified were pentane, hexanal, 2-pentylfuran, *trans*-2,*cis*-4-decadienal, and *trans*-2,*trans*-4-decadienal. These products originate from decomposition of either the C-9 or C-13 hydroperoxides generated by peanut lipoxygenase.

INTRODUCTION

Peanut lipoxygenase can react with linoleic acid or its methyl ester to form the C-9 and C-13 hydroperoxides (1). Once these products are formed, they can be further oxidized to secondary products, such as alcohols, acids, ketones, and aldehydes that can affect flavor and react with amino acids or protein to lower the nutritive value of the peanuts.

In 1971, Dupuy and associates (2) reported a simple, rapid, highly sensitive (ppb) method for the analysis of volatiles from vegetable oils. Essentially, this method consisted of placing an oil sample directly into a heated injection port, sweeping the volatiles onto the head of a warm (40-70 C) column, and resolving the volatiles by temperature-programmed gas chromatography (3-6). The method was used to analyze the volatiles from raw and roasted peanuts (3), peanut butter (7), salad oils and shortenings (5), and vegetable oils (6,8,9). This method was also used to study the products from the action of peanut lipoxygenase on linoleic acid. That report suggests that the C-13 position was the only point of attack by the enzyme (10).

Since that communication Dupuy and colleagues have made several improvements on the original method and have used this revised technique for determining flavor and flavor stability of fats, oils, seafoods, and cereal grains (11-15). These improvements were (1) a change in the absorbent material used in the column, (2) an external heated inlet-condenser assembly, equipped with a six-port rotary valve, which offers greater flexibility in the working conditions, and (3) a mass spectrometer interfaced with a computer. This system allows for gas chromatographic (GC) analysis and identification by combined direct GC and mass spectrometry (GC-MS) of volatiles produced from fatty acid oxidation without any preparation or chemical modification of samples.

In this paper, we discuss results obtained with the improved direct GC and combined direct GC-MS procedures on the analysis of products derived from linoleate oxidation catalyzed by peanut lipoxygenase under varying conditions. This work was initiated to understand better the action of lipoxygenase on linoleic acid in peanuts and is part of our continued research on origin of flavors in peanut and peanut products.

EXPERIMENTAL PROCEDURES

Materials

Peanuts (Virginia 56-R) were obtained from a commercial seed supplier in Holland, VA. Linoleic acid was purchased from Calbiochem, San Diego, CA, methyl linoleate from three different commercial suppliers, and Tenax GC, 60-80 mesh (2,5-diphenyl-paraphenoxylene oxide), and Poly MPE (poly-metaphenoxylene) from Applied Science Laboratories, State College, PA.

Substrate Preparation

One gram of linoleic acid or the methyl ester was added to 200 ml of a solution containing sodium phosphate buffer, pH 6.2, 0.1 M, and 0.4 ml Tween-20. The mixture was sonicated for several seconds into a one-phase suspension, separated into 20-ml portions, flushed with nitrogen, stoppered, and stored at -20 C until used.

[1]Use of a company of product name by the Department does not imply approval or recommendation of the product to the exclusion of others, which may also be suitable.

Enzyme Preparation

Lipoxygenase was prepared from raw peanuts as previously described (16). Briefly, the peanuts were hand-blanched, then homogenized with deionized water (5 ml/g) with an Omnimixer with 3-15 second alternating high-speed revolutions at 0 C. The homogenate was strained through 8 layers of cheesecloth and then centrifuged twice at 17,000 x g for 15 min and at 4 C. The supernatant was used as the source of enzyme.

Enzyme Assay

Before tests with linoleic acid were begun, enzyme preparations were monitored for lipoxygenase activity by measuring oxygen uptake as previously described (17). The initial dissolved oxygen concentration was taken as 240 nmoles/ml at 25 C. For GC-MS assay, the enzyme reaction medium consisted of 20 ml of substrate suspension and 4 ml of the enzyme preparation (28 mg protein/ml). Reactions were stirred up to 1 hr at 25 C in an open system with constant oxygen bubbling or in a closed system with no added oxygen. In the latter case, the oxygen content was ca. 6 μmoles/24 ml of reaction medium. At a given interval, samples were withdrawn (0.2 ml) and injected directly into the glass liner of the external inlet attached to the GC-MS instrument. Controls, consisting of the substrate medium or an enzyme-buffer solution, were also run through the direct inlet GC-MS system. Protein was determined by the Lowry method (18).

Instrumentation

A Tracor model 222-GC interfaced with a Hewlett-Packard (Quadrapole) mass spectrometer, model 5930A, was used to determine the volatiles from reaction mixtures. The ionization potential of the mass spectrometer was 70 eV and the scan range was from 33-450 A.M.U. Data processing was accomplished with an INCOS 2000 mass spectrometer data system. Except for temperature programing, the combined GC-MS and closed external assembly inlet system used were as described by Legendre et al. (12). The inlet temperature was generally 120 C, detector was at 250 C, and column oven was maintained at 30 C during the initial 20-min hold period. After removal of inlet liner, the column was heated to 100 C within 2 min and programmed for 5 C/min for 24 min. Final hold was at 220 C for 15 min.

RESULTS AND DISCUSSION

Although direct GC and combined direct GC-MS offer no special advantage over conventional methods for headspace analysis they offer a practical approach for the analysis and identification of volatile components found in the solvent phase of enzymatic reaction mixtures, as shown in Figure 1. In the closed system (1A), several components were observed with pentane and hexanal being predominent (ca. 35% and 30%, respectively). Other major volatiles produced in sufficient quantities for identification by mass spectrometry were 2-pentylfuran and two 2,4-decadienals. Upon examination of 10 ml of the headspace from the closed system, only 2 components were observed, pentane and hexanal. These results confirmed the findings of Pattee et al. (19), who showed pentane and hexanal to be the major volatile end-products found in the headspace of a peanut lipoxygenase-linoleate model system.

When the open system (1B) was examined (continuous purging for 1 hr with oxygen), the concentration of the volatiles observed in the closed system were greatly increased. Additionally, other volatiles were also observed: pentanal, pentanol, and 2-heptanone. Whereas maintaining an inlet temperature of 120 C is sufficient to produce volatiles, raising the inlet temperature to 140 C or to 160 C increased the quantities of the volatiles eluted. Each of these volatile components has been previously

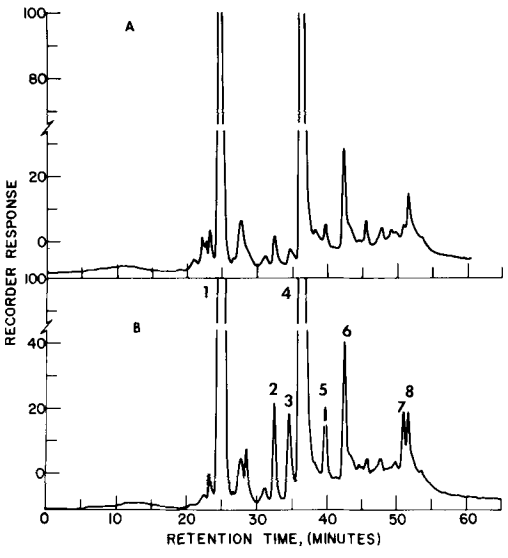

FIG. 1. Direct GC profile of volatiles observed in aqueous phase from linoleic acid-lipoxygenase reaction mixture. A represents those found in the closed system; B, volatiles from reaction run in the open system with constant oxygen purging. 1, pentane; 2, pentanal; 3, pentanol; 4, hexanal; 5, 2-heptanone; 6, 2-pentylfuran; 7, *trans*-2,*cis*-4-decadienal; 8, *trans*-2,*trans*-4-decadienal.

identified as a product from lipid oxidation of various plant materials by different laboratories using time-consuming conventional methods such as solvent extraction, distillation, and chemical modification (19-28).

In conventional GC procedures, the volatile extracts are normally injected into a heated injection port and flushed through the column with a carrier gas. The closed external inlet-condenser assembly offers greater flexibility in the analysis of a total aqueous reaction mixture. The results of an experiment demonstrating another advantage is shown in Figure 2. The aqueous reaction medium from a closed system was analyzed by sweeping the sample through the external inlet port at a temperature of 25 C throughout the 20 min sweeping period. After depositing any volatiles onto the column, the inlet is isolated from the GC by the 6-port valve, and the column is then temperature programmed. By this procedure, only hexanal was positively identified as a product; no other volatile compounds were identified. The small volatiles that were eluted prior to hexanal were in such small concentrations (less than 0.05 ppm) that they were not identified by MS.

While the volatiles were being run through the GC-MS, the condenser is being heated to drive off any moisture collected. After the run was completed and the entire system was cooled to room temperature, the spent sample was placed back into the inlet port, the temperature was increased to 120 C, the volatiles were then swept from the inlet onto the column, and finally, the GC was again temperature programmed. Figure 2B shows a chromatogram of the volatiles eluted at the elevated temperatures. This chromatogram was similar to results found in the reaction medium shown in Figure 1A, except that the *trans,cis*-decadienal was eluted at a low concentration.

Because hexanal was the only volatile component found in the room temperature system (noting that the enzymatic reaction was run at room temperature and that the reaction medium was analyzed with the inlet port never exceeding room temperature), these results suggest that the other volatile components observed in chromatogram 2B resulted from thermal decomposition of the hydroperoxides formed. These data support the work of Evans et al. (29), who showed that pentane is obtained from thermal decomposition of purified 13-hydroperoxyoctadeca-9,11-dienoic acid. They further concluded that pentane is the single major hydrocarbon component derived from thermal breakdown of the polar-polymeric product that results from lipid

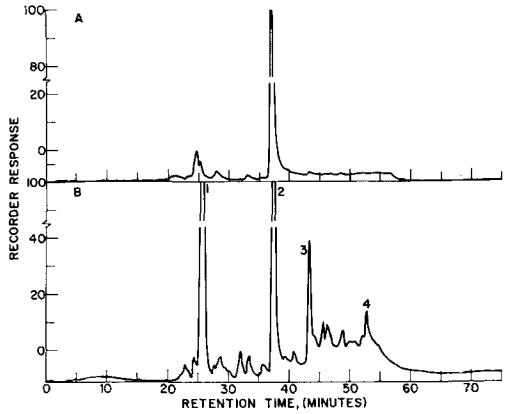

FIG. 2. Direct GC profile of volatiles observed in aqueous phase from linoleic acid-lipoxygenase reaction mixture run in closed system. Chromatogram A represents volatiles eluted from a sample with the inlet port at a temperature of 25 C, Chromatogram B, inlet port temperature was 120 C. 1, pentane; 2, hexanal; 3, 2-pentylfuran; 4, *trans*-2,*trans*-4-decadienal.

oxidation. Using the direct GC-MS method, we have now shown that other compounds are also derived in a similar manner. By only the headspace analysis and similar GC methodology, which included a heated inlet, pentane was proposed to be lipoxygenase mediated in soy and peanuts (19,30). Our data were obtained from aqueous samples injected into a cold (25 C) closed external assembly inlet system. The volatiles were swept from the sample and deposited on the head of the GC column, where they were resolved. Pentane, not observed from analysis of the aqueous phase before heating, seems to have resulted from thermal decomposition.

Other experiments were conducted as controls. Hexanal (1 ppm) was injected directly into the external inlet and analyzed with the inlet temperature at 25 C. Only hexanal was eluted; pentane was not observed. When the spent sample was placed back into the inlet port and the temperature raised to 120 C, no products were eluted, suggesting that all the hexanal was stripped from the sample tube at 25 C. Hexanal was next injected into the external inlet and analyzed at 120 C. Again, only hexanal was eluted; pentane was not. When a linoleic acid control was analyzed with the inlet set at 120 C, no products were eluted. These results suggest that in the aqueous reaction mixtures, pentane was not a product of thermal decomposition of hexanal or of linoleic acid, but of the hydroperoxide (as shown in Fig. 2).

To test the possibility that the enzyme could react with hexanal to form pentane, hexanal

was incubated with peanut lipoxygenase for 1 hr with constant oxygen purging. When a portion of the reaction mixture was injected into the external inlet set at 120 C, only hexanal was eluted. Again, no pentane was observed. These controls showed that the enzyme does not, as expected, react with hexanal to form pentane, nor is pentane formed from the thermal decomposition of hexanal at the lower temperatures used in these tests.

The aqueous reaction medium from a closed system with methyl linoleate as substrate was also examined with the inlet port at room temperature (Fig. 3A). Hexanal was again the only volatile compound that could be attributed directly to the enzymatic reaction in the aqueous phase. The other volatile components eluted between 30 and 35 min were identified as acetone and chloroform, probably minor contaminants retained during the preparation of the methyl linoleate. Furthermore, the methyl ester controls (no enzyme) yielded only acetone and chloroform volatile components when run at room temperature. No pentane was observed. Samples of methyl linoleate from three commercial preparations were analyzed, and some minor contaminants were found in each.

Upon completion of the volatile analysis at room temperature, the spent sample from 3A was flushed through the column and reexamined at 120 C. Results (3B) show that in addition to hexanal other volatiles are observed: pentane, 2-pentylfuran, methyl octanoate, and the 2,4-decadienals.

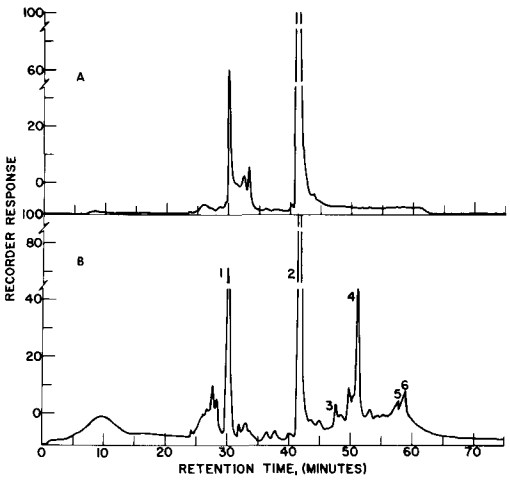

FIG. 3. Direct GC profile of volatiles observed in aqueous phase from methyl linoleate-lipoxygenase reaction mixture. Conditions were same as in Figure 2. 1, pentane; 2, hexanal; 3, 2-pentylfuran; 4, methyl octanoates; 5, *trans*-2,*cis*-4-decadienal; 6, *trans*-2,*trans*-4-decadienal.

To propose the possible origin of the identified decomposition products obtained from either the C-13 or C-9 hydroperoxides of linoleic acid or the methyl ester, the following schemes are offered (cleavage at points A, B, C, and so forth, produces the product listed):

1. CH_3-$(CH_2)_4$-CH(OOH)-CH=CH-CH=CH-$(CH_2)_7$-COOR*
   A B C
   A. Pentane, pentanal, pentanol.
   B. Hexanal
   C. 2-Heptanone.

2. CH_3-$(CH_2)_4$-CH=CH-CH=CH-CH(OOH)-$(CH_2)_7$-COOR*
   D E
   D. 2-Pentylfuran.
   E. t,t-2,4-Decadienal, t,c-2,4-decadienal, methyl octanoate.

*R, either H or CH_3.

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Sterol Composition of Nystatin and Amphotericin B Resistant Tobacco Calluses

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ABSTRACT

The objective of this study was to select and characterize tobacco cell lines resistant to the polyene antibiotics Amphotericin B and Nystatin. Suspension culture cells of tobacco (*Nicotiana tabacum* L. 'Wisconsin 38') were treated with 0.025% ethyl methane sulfonate (EMS) and suspended in melted agar medium containing 0.005 mg/ml Amphotericin B. Cells were also irradiated with UV from a germicidal lamp and plated in the melted agar plating medium. After 6-10 days growth, the cultures were overplated with medium containing 750 u/ml Nystatin. All cultures were grown at 26 C in the dark. After three weeks, a single callus was isolated from the Amphotericin B treatment. This callus was subcultured, and would grow at concentrations of the antibiotic as high as 0.1 mg/ml (a 20-fold increase over the selection concentration). Six callus lines were isolated from the Nystatin treatment. After subculture and transfer to media containing higher concentrations of Nystatin, only two calli survived and would grow at 1250 u/uml. Gas liquid chromatographic analysis of the resistant and control callus lines revealed similar sterol content among all the lines. However, when comparing total sterols produced, all three polyene resistant calluses contained significantly more sterols than the control. The Amphotericin B resistant calli produced about three times as much sterol as the controls, and the Nystatin resistant calli produced about 1.5 times as much sterol as the control. Both Nystatin resistant lines showed significant increases in stigmasterol. It appears that resistance of the three tobacco cell lines to these polyene antibiotics is due principally to varied amounts of overproduction of sterols rather than qualitative differences in sterols.

INTRODUCTION

The polyene antibiotics, Amphotericin B and Nystatin, are widely used for the treatment of fungal infection. However, animal and plant cells, in addition to fungi, are also found to be sensitive to these drugs (1-3). The inhibitory effect of the polyene antibiotics is believed to result from the formation of complexes with sterols in the cell membrane. The result of this interaction is altered membrane permeability and eventual death of the cells (4). Artificial membrane studies indicate that the rate of association and interaction between polyenes and membrane sterols closely relates to the ratio of phospholipid/cholesterol (5-8), and furthermore, the degree of binding depends on both the absolute concentration of cholesterol and on the mol % of cholesterol in the bilayer (9). Polyene antibiotics are also reported to be inhibitory to photosystem 1 activity by changing the chloroplast membrane permeability and releasing plastocyanin from its site in the photosynthetic electron transfer chain (5).

Induced mutation in plant cells has been reviewed by Maliga (10). So far, mutants resistant to polyene antibiotics have been reported in many organisms (11-15), but not in higher vascular plants. Most commonly, resistant strains contain replacement sterols which have less affinity for the polyene than wild type membrane sterols.

In this paper, an attempt has been made to isolate dark-grown tobacco tissue culture cell lines resistant to Nystatin and Amphotericin B. Since all of the sterols which are structural components in the cytoplasmic membrane are free sterols, an investigation of the free sterol composition and its correlation with resistance to polyene antibiotics is reported.

MATERIALS AND METHODS

Tobocco Tissue Cultures

Callus derived from pith explants was used to initiate suspension cultures of tobacco (*Nicotiana tabacum* L. 'Wisconsin 38'). The suspension cultures were maintained as 100 ml cultures, in 250 ml Erlenmeyer flasks on a gyrotary shaker at 120 rpm in the dark at 25 C. The cells were subcultured twice weekly. The medium of Murashige and Skoog (16) at pH 5.7 with 0.5 mg/l 2,4-dichlorophenoxyacetic acid was used to maintain the cell suspensions.

Determination of Inhibitory Concentrations of Amphotericin B and Nystatin

Cells from a culture two days after subculturing were harvested by filtration through one layer of cheese cloth. The filtrate was centrifuged at 100 rpm for 5 min. After centrifugation, the supernatant was poured off the the cells resuspended in 30 ml of melted plating

medium containing various concentrations of one of the polyene antibiotics. The plating medium consisted of the salts, iron chelate, agar and organic compounds of Murashige and Skoog (16) with 30 g/l sucrose, 2 mg/l naphthalene acetic acid, and 0.65 mg/l kinetin at pH 5.6. The contents of the tube were divided between 10 cm petri dishes. After the agar had solidified, the petri dishes were wrapped with aluminum foil and placed in an incubator at 26 C in the dark. The concentration series of the antibiotics used were: a five-fold dilution of Amphotericin B from 0.00005 mg/ml to 1 mg/ml, and two-fold dilutions from Nystatin from 375 u/ml to 3000 u/ml. Two to three weeks after plating, growth of the cells was observed. The lowest drug concentration at which complete inhibition of growth occurred was used as the concentration for selection of resistant cell lines.

Isolation of Resistant Lines – Amphotericin B Resistance

Cells were filtered and centrifuged as above. Twenty ml of the MS medium containing 0.05 ml of ethyl methane sulfonate (EMS) giving a final EMS concentration of 0.025% was added to the pellet of cells. This concentration of EMS had been previously shown to produce 50% cell survival. The cells were treated with EMS on a shaker at 120 rpm for 2.5 hr. At the end of the mutagen treatment, the cells were washed three times in fresh MS medium. After the third wash, the cells were suspended in melted plating medium containing 0.005 mg/ml Amphotericin B. This concentration was the lowest giving 100% cell mortality. The melted agar-cell suspension was poured into petri dishes. After the agar had solidified, the cells were placed in an incubator in the dark at 26 C.

Nystatin Resistance

Fifteen ml of filtered tobacco cells were placed in a 10 cm petri dish and exposed to two 15 W germicidal lamps 10 cm above the dishes for 1.5 min. The cell suspension was constantly stirred with a magnetic stirrer to insure a uniform exposure. This treatment had been shown to reduce cell survival of 37%.

After UV treatment, the cells were returned to a flask, and placed on the shaker (120 rpm) for 6 days. The cells were then collected by centrifugation as before, suspended in the melted agar plating medium and poured into petri dishes. The cultures were placed in an incubator in the dark at 26 C for 6-10 days. At this point, the cultures were over-plated with 20 ml of the plating medium containing 750 u/ml Nystatin (the lowest concentration found to give 100% cell killing). The cultures were again returned to the incubator in the dark at 26 C.

Sterol Analysis of Resistant Calluses

The resistant calluses were collected, frozen, and freeze dried. Dry calluses were ground into powder, and the lipid extracted with methanol/chloroform (1:2) in a Soxhlet for 24 hr. The extracting solvent was evaporated and the lipid redissolved in a small amount of chloroform. The chloroform soluble fraction was filtered, the filtrate was evaporated and the total lipid obtained. The total lipid was then chromatographed on Silica Gel G thin layer plates with hexane/diethylether/acetic acid (80:20:1) as the developing solvent. The separated components were detected after spraying 0.1% Dichlorofluorescein under a UV lamp. The free desmethyl sterol band was separated visually from other components by comparing the Rf with a cholesterol standard. The free desmethyl sterols were scraped from the plate, eluted with chloroform and recovered by evaporation of the solvent at room temperature in a stream of nitrogen.

The sterol mixture was analyzed on a Varian Model 3700 gas liquid chromatograph with a Varian CDS 111 and Model 9176 recorder, glass column packed with 1% SE-30, and using the following conditions: column, 225 C; injector, 250 C; flame ionization detector, 300 C; flow rate of carrier gas, 30 ml/min.

RESULTS AND DISCUSSION

After a three-week growth period, a single callus (FGI) was isolated from the Amphotericin B treatment. The isolated callus was transferred to the surface of solidified plating medium for growth. Over succeeding transfers, the calli were subjected to increasing concentrations of Amphotericin B. After several transfers, the callus showed resistance to Amphotericin B at concentrations of 0.1 mg/ml which is a 20-fold increase over the original selection medium.

Six callus lines resistant to Nystatin (NY1, NY2, NY3, NY4, NY5, NY6) were isolated from the UV-irradiated cells. After transfer of resistant calli to increasing concentrations of Nystatin containing plating medium, four failed to grow on 1250 u/ml Nystatin. Only two calli continued their growth on this highly concentrated Nystatin medium. They were subdivided and propagated continuously on Nystatin. The two resistant calli (NY3, NY4) were originally the first to emerge from the selection medium. It is very likely that the

four which emerged later were grown in selection medium where the Nystatin had decomposed due to the long period of incubation, since polyene antibiotics are light- and heat-sensitive compounds. Since it takes about two weeks to observe callus growing on the plating medium when using suspension cells, the concentration of polyene that is used for selection is much higher than that used in other reported experiments on fungi (12). Also, the high concentration of drug required in the selection medium could be due to the fact that the presence of the plant cell wall might somehow protect the plant cell membrane (3).

Gas liquid chromatographic analysis of the sterol contents of FGI, NY3, NY4, and control callus was obtained. The chromatographs (Fig. 1) indicate a similar sterol content among the resistant lines. When the percentage of each sterol is calculated (Table I), FGI has a sterol content very similar to the control callus, in which the predominant sterols are sitosterol and campesterol with a very small amount of cholesterol and stigmasterol. In NY4, 15.59% of the total desmethyl sterol fraction is stigmasterol, in comparison with the control which has only very small amounts of stigmasterol. NY4 was the first resistant callus to emerge from the Nystatin selection medium and always showed comparatively a more rapid growth rate than NY3 on drug medium.

When comparing the amount of total sterol produced (Table II), all three polyene resistant calluses contain significantly more sterols than the control. The EMS-treated Amphotericin B (FGI) resistant callus is the most notable, producing about three times the amount of sterols as the control. The other two resistant calluses also produce almost 1.5 times as much sterol as the control.

In previous studies, polyene antibiotic-resistant mutants have been isolated from *Cryptococcus neoformans* (13), *Sacharomyces cerevisiae* (11,14,17-19), *Candida albicans*

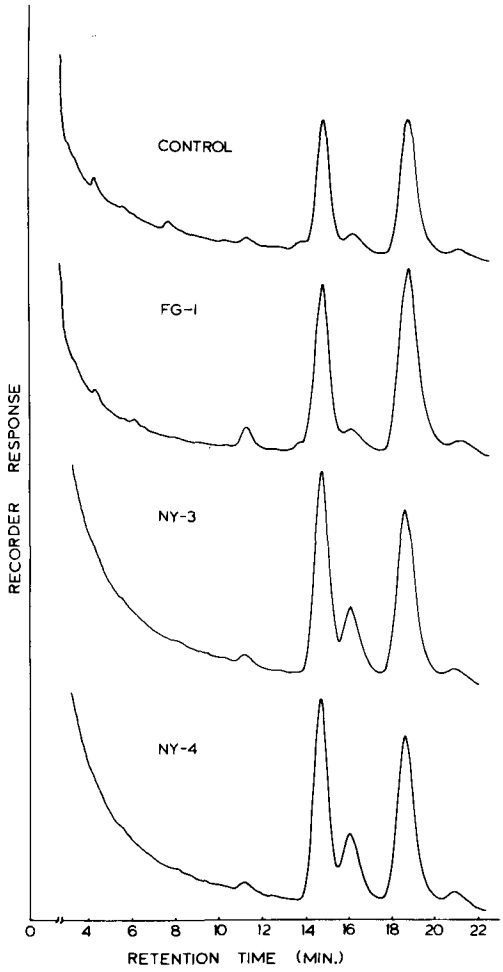

FIG. 1. Gas chromatograms of sterols from control, FG-1, NY-3, NY4-calli. The retention times of cholesterol, campesterol, stigmasterol and sitosterol are 11.1, 14.5, 15.9, and 18.5 minutes, respectively.

TABLE I

Percentages of Isolated Sterols in Control and Polyene Resistant Tobacco Calluses

Resistant line	Sterol			
	Cholesterol	Campesterol	Stigmasterol	Sitosterol
Control	1.18	36.37	7.07	51.86
FG1	2.72	34.37	6.16	50.40
NY3	0.59	36.55	15.82	41.36
NY4	1.64	36.29	15.59	39.16

TABLE II

Comparison of Total Free Sterol Isolated from Drug Resistant and Control Calluses

Resistant line	Dry wt. (g)	Total free sterol (mg)	Sterol/D.W. (%)
Control	1.6	2.6	0.16
FG1	0.7	3.5	0.50
NY3	0.8	2.2	0.27
NY4	1.8	4.1	0.23

(20,21) *Candida tropicalis* (15), and *Neurospora crassa* (12,22). All of the above organisms contain ergosterol in their wild type mycelium. The sterol composition of the mutant strains usually falls into one of the following categories: (a) Ergosterol was present in a reduced amount; (b) one or more sterol intermediates have replaced ergosterol and become the predominant sterol; (c) sterol synthesis has been completely inhibited and the mutant strain requires exogenous sterol or fatty acid for growth; and (d) the altered sterol patterns contain significantly more sterol than did the sensitive parent strain. The changes indicate that the sensitivity of cells containing different sterols vary in their sensitivity to antibiotics in the following order: $\Delta^{5,7}>$, $\Delta^{7}>$, $\Delta^{8(9)}$-sterol.

In tobacco, there is no ergosterol or any $\Delta^{5,7}$-sterol present. The two major sterols, sitosterol and campesterol, are Δ^{5}-sterols. Based on experimental data, there is no indication of which particular sterol more effectively binds with Amphotericin B in tobacco cells. The binding of Amphotericin B to the sterols may be evenly effective, so the resistance is due to overproduction rather than qualitative differences in sterols. Mutants isolated from *C. neoformans* also exhibit high resistance to Amphotericin B, but have a sterol composition identical to that of the wild type. In some cases, the mutants lacking ergosterol are still sensitive to Amphotericin B (13).

Whether Nystatin resistance is due to the same mechanism as Amphotericin B, resistance is very difficult to conclude based on the data here. However, Amphotericin B resistant mutants have been demonstrated which are still sensitive to Nystatin (13). Nystatin was suggested to be the most effective polyene antibiotic to select the greatest variety and highest proportion of sterol mutants (21) due to the fact that it effectively binds to ergosterol (13). In addition, the less effective binding of Nystatin to $\Delta^{7,22}$-ergostadien-3β-ol was suggested (13).

The Nystatin resistant calluses, NY3 and NY4 showed significant alteration of sterol composition by their increased stigmasterol. This may suggest that stigmasterol provides a stabilizing effect on membranes due to its less effective binding to Nystatin.

It therefore appears that resistance of the three tobacco cell lines to the polyene antibiotics is due principally to varies amounts of over-production of sterols, rather than qualitative differences in sterols between sensitive and resistant lines. Since we have not regenerated plants from any of the resistant calli and established the genetic nature of the resistance, the variants cannot be classified as true mutations. Only indirect evidence of the altered level of sterols in the resistant lines is available so far. This is very similar to lines of tobacco and carrot resistant to amino acid analogs (23). Resistance is attributed to overproduction of the normal amino acid due to altered feedback sensitivity of an enzyme in the pathway for amino acid synthesis. Presumably, in the polyene antibiotic resistant lines, excess sterol would bind the antibiotic and thereby prevent it from binding with membrane sterols.

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Reduction of Essential Fatty Acid Deficiency in Rats Fed a Low Iron Fat Free Diet

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ABSTRACT

Young male rats were fed ad libitum for 8 weeks a low iron fat-free (FF-Fe) diet or a fat-free diet supplemented with iron (FF+Fe). The relative levels of 16:1 to 16:0 and 18:1 to 18:0 in the total fatty acids of liver and other tissues (plasma, erythrocytes and intestinal mucosa) were considerably decreased because of a lack of dietary iron. In rats fed the FF-Fe diet, the levels of essential fatty acids ($18:2\omega6$ + $20:4\omega6$) in tissues were 2- to 3-fold greater than in the corresponding tissues of rats fed the FF+Fe diet. Eicosatrienoic acid ($20:3\omega9$) levels in tissue lipids from rats fed the FF+Fe diet were high (8-16%), whereas they were low (2-5%) in the case of animals fed the FF-Fe diet. The proportion of 20:4 in total fatty acids of tissues was 2- to 3-fold greater in rats fed the FF-Fe diet than when they were fed the FF+Fe diet. Therefore, the relative levels of $20:3\omega9/20:4\omega6$ varied from 1-2.9 in tissue lipids of rats fed the FF+Fe diet, while it varied only from 0.2-0.3 in animals fed the FF-Fe diet. These results suggest that a lack of dietary iron may reduce the synthesis of 16:1, 18:1, 20:3 and 20:4 and the metabolism of 20:4.

INTRODUCTION

Stearoyl CoA desaturase enzyme complex consists of NADH-cytochrome b_5 reductase, cytochrome b_5, lipids and the terminal desaturase enzyme which is a non-heme iron protein (1-3). Numerous investigations have dealt with the changes in the tissue desaturase activity due to dietary factors such as fat (4-11), cyclopropene fatty acids (12-14) or to pathologic conditions such as diabetes (15,16), obesity (17), phenyl ketonuria (18) and neoplasia (8-11,19,20). However, to our knowledge, studies have not been carried out to determine the role of a lack of dietary iron on the desaturase activity. In the present study, we maintained rats on a fat-free diet, which is known to stimulate tissue desaturase activities in animals (4-11). The fat-free diet was either deficient in iron or supplemented with iron. We analyzed the fatty acid compositions of various tissue lipids of rats fed these two fat-free diets to determine whether these would indicate a decrease in the tissue desaturase activity stemming from a lack of dietary iron. Our results show that the desaturase activities involved in the synthesis of mono- and polyenoic acids may be depressed by feeding a diet deficient in iron. A preliminary report of this study has already appeared (21).

MATERIALS AND METHODS

Young male Sprague Dawley rats weighing 50 g were purchased from Hilltop Animal Supplier, Chatsworth, CA. A group of 4 rats were fed a fat-free diet containing 75 μg Fe/g diet ad libitum for 8 weeks. Another group of 4 rats were fed a fat-free diet having a low iron content (10 μg Fe/g diet). Rats were kept in plastic cages having plastic ventilated covers. They had free access to distilled water fed through glass sipper tubes. Sawdust bedding in the cages was changed twice a week. Diets were obtained from ICN Nutritional Biochemicals, Cleveland, OH. Fat-free diet is a standard catalog item whose composition is given in the *ICN diets manual*. The low iron fat-free diet was custom made with regular dietary components and elimination of the ferrous ammonium citrate from the U.S.P. salt mixture XIV. The iron content of the diet was determined spectrophotometrically (22) using Ferro Zine (3-[2 pyridyl]-5,6-bis[4-phenyl sulfonic acid]-1,2,4-triazine) with $FeSO_4$ as a standard. Ferro Zine was obtained as iron color reagent (Technicon No. T01-0515) from Technicon Instruments Corporation, Tarrytown, NY.

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/ml/300 g rat) and exsanguinated using heparin-washed syringes and needles. Whole blood was transferred without pressure into heparinized vacutainer tubes. Blood was centrifuged at ca. 22 C at 2500 rpm for 7 min and plasma and buffy layer were removed. Red cells were washed three times by suspension in standard incubation media (23). Hemoglobin determinations were carried out using the cyanmethemoglobin method. The packed red cell volumes were measured using a Phillips-Drucker hematocrit centrifuge and read on an IEC microcapillary reader.

Immediately after the removal of blood, livers were excised, blotted and washed with ice cold saline. The upper half of the intestine was removed and freed from mesentric tissue. Using a syringe, ice cold saline was forced through the intestine several times to remove the intestinal contents. The intestine was slit open on a glass plate kept over ice and the mucosa was re-

TABLE I

Changes in the Fatty Acid Composition of Liver Lipids Due to the Reduction of the Iron Content of Fat-Free Diet Fed to Rats[a]

	Total lipid		Phospholipid		Triglyceride	
Fatty acid	FF+Fe	FF-Fe	FF+Fe	FF-Fe	FF+Fe	FF-Fe
16:0	24.4 ± 0.6	21.9 ± 0.4	18.5 ± 0.3	19.3 ± 1.2	32.5 ± 1.1	31.3 ± 0.8
16:1ω7	14.1 ± 0.5	7.2 ± 0.1	8.9 ± 0.5	6.4 ± 0.4	17.9 ± 0.5	12.6 ± 0.7
18:0	11.3 ± 0.6	18.8 ± 0.1	23.8 ± 0.6	24.2 ± 0.7	3.7 ± 0.2	3.8 ± 0.2
18:1(ω9+ω7)	30.1 ± 0.3	22.7 ± 0.5	21.6 ± 0.6	16.9 ± 0.7	45.8 ± 1.4	48.8 ± 0.5
18:2ω6	2.2 ± 0.2	6.9 ± 0.7	3.4 ± 0.3	11.4 ± 0.3	0	3.5 ± 0.3
20:3ω9	9.1 ± 0.7	4.0 ± 0.3	13.1 ± 0.5	3.9 ± 0.2	0	0
20:4ω6	5.6 ± 0.6	14.7 ± 0.4	7.9 ± 0.6	13.9 ± 0.6	0	0
22:4ω6[b]	1.4 ± 0.1	2.0 ± 0.2	1.2 ± 0.1	1.7 ± 0.1	0	0
22:6ω 3	1.4 ± 0.2	1.9 ± 0.1	1.1 ± 0.1	1.8 ± 0.2	0	0
$\frac{16:1}{16:0}$	0.57 ± 0.01	0.33 ± 0.01	0.50 ± 0.03	0.34 ± 0.03	0.55 ± 0.01	0.40 ± .03
$\frac{18:1}{18:0}$	2.72 ± 0.18	1.31 ± 0.08	0.91 ± 0.35	0.70 ± 0.04	12.6 ± 0.6	13.0 ± 0.7
$\frac{18:1+20:3}{18:0}$	3.53 ± 0.19	1.42 ± 0.04	1.46 ± 0.05	0.86 ± 0.05	---	---
$\frac{20:3\omega 9}{20:4\omega 6}$	1.62 ± 0.06	0.27 ± 0.03	1.70 ± 0.17	0.28 ± 0.01	---	---
18:2+20:4[c]	7.8 ± 0.6	21.6 ± 0.8	11.3 ± 0.6	25.3 ± 0.7	0	3.5 ± 0.3

[a]Percent of total fatty acids given as Mean ± SE of separate analysis with liver samples from four rats fed the FF+Fe or FF-Fe diets for 8 weeks. Values for 14:0 were not obtained. Methyl ester of this acid eluted with butylated hydroxytoluene (BHT) during the gas liquid chromatographic (GLC) separation.

[b]Includes 24:0 and 24:1ω9.

[c]Percent of total fatty acids.

TABLE II

Changes in the Fatty Acid Composition of Plasma Lipids Due to the Reduction of the Iron Content of Fat-Free Diet Fed to Rats[a]

	Total lipid		Phospholipid		Triglyceride	
Fatty acid	FF+Fe	FF-Fe	FF+Fe	FF-Fe	FF+Fe	FF-Fe
16:0	19.9 ± 0.4	21.1 ± 0.8	20.7 ± 1.0	24.8 ± 1.0	31.1 ± 1.0	29.6 ± 0.9
16:1ω7	14.0 ± 0.5	8.7 ± 0.4	4.4 ± 0.3	2.4 ± 0.3	16.3 ± 0.9	7.6 ± 0.6
18:0	10.9 ± 0.4	12.2 ± 0.4	22.9 ± 1.4	22.4 ± 0.8	5.7 ± 0.6	10.2 ± 0.6
18:1(ω9+ω7)	32.1 ± 0.5	24.8 ± 1.0	26.2 ± 1.2	17.7 ± 0.8	45.6 ± 2.3	47.1 ± 1.7
18:2ω6	3.2 ± 0.1	11.7 ± 0.5	2.3 ± 0.1	12.6 ± 0.5	1.3 ± 0.2	5.6 ± 0.6
18:3ω3	T	1.6 ± 0.1	0.7 ± 0.1	T	---	---
20:3ω9	14.0 ± 0.6	5.0 ± 0.6	14.4 ± 2.2	4.0 ± 0.4	---	---
20:4ω6	5.3 ± 0.3	14.8 ± 0.7	5.3 ± 1.2	12.9 ± 1.1	---	---
22:4ω6[b]	---	---	1.9 ± 0.2	1.2 ± 0.1	---	---
22:6ω3	---	---	1.1 ± 0.1	1.6 ± 0.1	---	---
$\frac{16:1}{16:0}$	0.70 ± 0.04	0.42 ± 0.03	0.21 ± 0.03	0.10 ± 0.02	0.53 ± 0.02	0.26 ± 0.2
$\frac{18:1}{18:0}$	2.96 ± 0.15	2.03 ± 0.05	1.15 ± 0.03	0.79 ± 0.03	8.29 ± 1.2	4.67 ± 0.38
$\frac{18:1+20:3}{18:0}$	4.26 ± 0.2	2.47 ± 0.12	1.79 ± 0.16	0.97 ± 0.01	---	---
$\frac{20:3\omega9}{20:4\omega6}$	2.81 ± 0.14	0.34 ± 0.04	2.86 ± 0.57	0.31 ± 0.04	---	---
18:2+20:4[c]	8.5 ± 0.2	26.5 ± 0.7	7.6 ± 1.3	25.5 ± 1.1	1.3 ± 0.2	5.6 ± 0.6

[a]Percent of total fatty acids given as Mean ± SE of separate analysis with plasma from four rats fed FF+Fe or FF-Fe diets for 8 weeks. Values for 14:0 were not obtained. Methyl ester of this acid eluted with BHT during GLC.

[b]Includes 24:0 and 24:1.

[c]Percent of total fatty acids.

moved by gently scraping with a spatula.

Total lipids of erythrocytes were extracted as described by Rose and Oklander (24). From liver, plasma, and intestinal mucosa, lipids were extracted by the method of Folch et al. (25). Phospholipid and triglyceride fractions were isolated from the total lipids by thin layer chromatography using the developing solvent systems described by Brown and Johnston (26) and Mangold (27), respectively.

Fatty acid methyl esters were prepared from total lipids, phospholipids and triglycerides and analyzed by gas liquid chromatography as described earlier (23).

RESULTS AND DISCUSSION

The effect of reducing the dietary intake of iron on the composition of fatty acids was examined by analyzing the lipids in liver, in circulation (plasma and erythrocytes) and in cells that have a rapid turn over (intestinal musoca) (Tables I-IV). A significant decrease in the relative levels of 16:1 to 16:0 and of 18:1 to 18:0 was observed in the lipids of tissues from rats fed the low iron diet. Stearoyl CoA desaturase, which produces 18:1 from 18:0, is also about equally active in the production of 16:1 from 16:0 (28). Hence, the reduction in the relative levels of 16:1 and 18:1 can be related to the decrease in the stearoyl CoA desaturase activity in the tissues of rats fed a low iron diet.

When rats were fed the FF-Fe diet, levels of 20:3ω9 in total fatty acids of tissue lipids were low as compared to the levels in rats fed the iron-supplemented diet (Tables I-IV). This may be due to a reduction of the level of the precursor acid (18:1ω9), and of the activities for chain elongation and desaturation required for the synthesis of 20:3.

Relative levels of essential fatty acids (18:2 ω6 + 20:4ω6) in the total fatty acids of rat tissues was 2- to 3-fold greater when the FF-Fe diet was fed. Increased levels of 18:2 may be due to a decreased level of the chain elongation and desaturase activities necessary for the synthesis of 20:4. On the other hand, enhanced levels of 20:4 could be due to a decreased metabolsim of this tetraenoic acid in rats fed a low iron diet. For example, arachidonic acid is the essential substrate for the production of endoperoxides and thromboxanes. Iron or heme is an essential cofactor for the peroxidase, lipoxygenase and cyclo-oxygenase enzymes involved in the formation of these compounds (29).

The ratio of the levels of 20:3ω9 to 20:4ω6 in tissue lipids serves as a measure of essential fatty acid deficiency in an animal (30). A triene/tetraene ratio of less than 0.4 indicates that the minimal essential fatty acid requirement of the animal has been met. In the present investigation, although two groups of rats were fed a fat-free diet for an identical period (8 weeks), the relative levels of 20:3ω9 to 20:4ω6 in tissue lipids varied from 1-2.9 in animals fed the iron-supplemented diet while they varied from only 0.2 to 0.3 in those fed the low iron diet. Thus, a satisfactory supply of essential fatty acids was available to rats even when they were fed a fat-free diet for 8 weeks when the iron content of the diet was reduced (Tables I-IV).

Iron deficiency is expressed by the reduction of packed red cell volume and hemoglobin content of blood. However, in the present study, although rats were fed the fat-free diet containing a low amount of iron, the hematocrit and hemoglobin content of blood were not significantly reduced. The hemoglobin content of blood in rats fed the FF+Fe diet was 15.8 g%, while in those fed the FF-Fe diet, it was 15.0 g%. Packed red cell volume of blood in rats fed the FF+Fe or FF-Fe diets were 45.0% and 43.5%, respectively. Thus, it would appear that even when desaturase levels could have reduced by feeding FF-Fe diet, the syn-

TABLE III

Changes in the Fatty Acid Composition of Erythrocyte Lipids Due to the Reduction of the Iron Content of Fat-Free Diet Fed to Rats[a]

Fatty acid	Total lipid	
	FF+Fe	FF-Fe
16:0	22.7 ± 0.4	25.5 ± 0.2
16:1ω7	4.4 ± 0.2	2.7 ± 0.2
18:0	13.5 ± 0.2	15.1 ± 0.6
18:1(ω9+ω7)	20.2 ± 0.3	15.4 ± 0.6
18:2ω6	1.7 ± 0.1	5.5 ± 0.6
18:3ω3	T	T
20:3ω9	14.8 ± 0.2	3.9 ± 0.3
20:4ω6	15.5 ± 0.5	24.1 ± 1.1
22:4ω6[b]	5.7 ± 0.3	6.6 ± 0.3
22:6ω3	1.1 ± 0.1	1.5 ± 0.2
$\frac{16:1}{16:0}$	0.19 ± 0.01	0.11 ± 0.01
$\frac{18:1}{18:0}$	1.5 ± 0.04	1.02 ± 0.05
$\frac{18:1+20:3}{18:0}$	2.59 ± 0.06	1.3 ± 0.04
$\frac{20:3\omega 9}{20:4\omega 6}$	0.96 ± 0.03	0.16 ± 0.003
18:2+20:4[c]	17.2 ± 0.6	29.5 ± 0.8

[a]Percent of total fatty acids given as Mean ± SE of separate analysis with erythrocytes from four rats fed either FF+Fe or FF-Fe diets for 8 weeks. Values 0.5% or less are given as T. Values for 14:0 were not obtained. Methyl ester of this acid eluted with BHT during GLC.

[b]Includes 24:0 and 24:1.

[c]Percent of total fatty acids.

TABLE IV

Changes in the Fatty Acid Composition of Lipids of Intestinal Mucosa Due to the Reduction of the Iron Content of Fat-Free Diet Fed to Rats[a]

	Total lipid		Phospholipid		Triglyceride	
Fatty acid	FF+Fe	FF-Fe	FF+Fe	FF-Fe	FF+Fe	FF-Fe
16:0	19.1 ± 0.2	21.0 ± 0.8	15.1 ± 0.4	19.8 ± 0.9	32.2 ± 0.8	33.1 ± 0.9
16:1ω7	11.7 ± 0.6	7.7 ± 0.4	8.8 ± 0.5	3.8 ± 0.2	15.4 ± 0.8	9.6 ± 0.4
18:0	12.3 ± 0.8	17.2 ± 1.0	20.9 ± 1.1	20.4 ± 0.2	6.1 ± 0.4	10.8 ± 0.6
18:1(ω9+ω7)	39.0 ± 1.2	31.2 ± 0.5	32.1 ± 1.3	24.3 ± 1.4	44.7 ± 0.5	40.7 ± 0.4
18:2ω6	3.8 ± 0.2	12.0 ± 0.9	4.4 ± 0.1	18.0 ± 1.6	1.6 ± 0.4	5.8 ± 0.5
18:3ω3	1.5 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0	0
20:3ω9	8.3 ± 0.2	1.8 ± 0.2	12.2 ± 1.0	2.1 ± 0.4	0	0
20:4ω6	4.3 ± 0.2	7.3 ± 0.4	5.4 ± 0.6	10.7 ± 0.6	0	0
$\frac{16:1}{16:0}$	0.61 ± 0.04	0.37 ± 0.04	0.59 ± 0.03	0.19 ± 0.01	0.48 ± 0.04	0.29 ± 0.03
$\frac{18:1}{18:0}$	3.22 ± 0.32	1.83 ± 0.09	1.55 ± 0.12	1.19 ± 0.07	7.41 ± 0.61	3.79 ± 0.23
$\frac{18:1+20:3}{18:0}$	3.9 ± 0.40	1.93 ± 0.1	2.13 ± 0.14	1.3 ± 0.06	---	---
$\frac{20:3\omega9}{20:4\omega6}$	1.93 ± 0.13	0.26 ± 0.03	2.27 ± 0.17	0.20 ± 0.03	---	---
18:2+20:4[b]	8.1 ± 0.1	19.4 ± 1.1	9.8 ± 0.6	28.7 ± 2.2	1.6 ± 0.4	5.8 ± 0.5

[a]Percent of total fatty acids given as Mean ± SE of Separate analysis with mucosa from four rats fed either FF+Fe or FF-Fe diets for 8 weeks. Values for 14:0 were not obtained. Methyl ester of this acid eluted with BHT during GLC.

[b]Percent of total fatty acids.

thesis of red cells was unaffected. Whether the contents of various iron-containing enzymes or proteins in tissues of rats fed the FF-Fe diet have decreased remains to be investigated.

In a study parallel to the present one, we also fed a group of young rats (50 g) for 8 weeks either a 15% corn oil diet containing 81 μg Fe/g diet (Co+Fe) or a 15% corn oil diet having only 12μg Fe/g diet (Co-Fe). The presence of fat and a low iron content in the diet depressed the growth of rats significantly. The final body weights of animals fed the FF+Fe diet (370 g) were only slightly greater than of those fed the FF-Fe diet (333 g). However, when animals were maintained on the Co-Fe diet, their weights were considerably lower (258 g) as compared to those on Co+Fe diet (414 g).

Unlike the case of FF-Fe diet, when rats were fed the Co-Fe diet, iron deficiency was evident from the examination of blood. The blood hematocrit values of rats fed Co+Fe and Co-Fe diets were 47.0% and 24.5%, respectively. The hemoglobin content of blood in rats in the Co+Fe diet group was 16.0 g% while in those of the Co-Fe group, it was 8.7 g%. Mechanisms by which iron deficiency is produced by the presence of corn oil in a low iron diet are not known at present. The turnover of iron in tissues may be enhanced by feeding the polyunsaturated fat. Furthermore, the intestinal absorption of iron from the low iron diets may be more efficient when the diet is free of fat.

It is known that the tissue levels of desaturase are reduced when animals are fed a corn oil diet (11). When rats were fed the Co-Fe diet, a further reduction of desaturase activity could have occurred. This was suggested by a decrease of the relative levels of 16:1 to 16:0 in tissue lipids of rats fed the Co-Fe diet as compared to those in rats fed Co+Fe diets. Since corn oil contained appreciable amounts of oleic acid (25% of total FA) and linoleic acid (62%), these acids were present in large amounts in tissue lipids. As would be expected, tissues did not contain 20:3 when rats were fed the 15% Co diets.

It was shown recently that the reduced stearoyl-CoA desaturase activity in diabetic animals is due to decreased levels of the terminal desaturase enzyme (16). On the other hand, the lack of desaturase activity of Novikoff hepatoma is due to the absence of both cytochrome b_5 and terminal desaturase enzyme (20). The data presented in this paper suggest that the desaturase activities may be reduced by feeding the low iron FF diet. Further experiments on the determination of desaturase activities of tissues and the quantitation of the various components of the enzyme complex (11,20) are needed to elucidate the role of dietary iron in the production of mono and polyenoic fatty acids.

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METHODS

Improved Separation of Phospholipids in Thin Layer Chromatography

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ABSTRACT

The mobile phases described permit separation of the six major phospholipids of amniotic fluid in one dimension with either conventional or high performance thin layer chromatography. An example of this separation with an extract of amniotic fluid is given.

INTRODUCTION

Recent renewed interest in the separation of the phospholipids has accrued from its utility in the assessment of fetal lung maturity (1). Methodology in use at present for separation of the phospholipids in amniotic fluid, for complete separation of the six major components, involves two dimensional thin layer chromatography (1,2). We recently reported the separation of these compounds in one dimensional thin layer chromatography (TLC) which included complete separation of phosphatidylglycerol (PG) (3). Since phosphatidylglycerol traveled close to some of the other phospholipids, an investigation of the use of other mobile phases and the use of high performance thin layer chromatography (HPTLC) was carried out. This report describes the development of improved mobile phases to increase the separation of PG and also the more rapid separation accomplished by HPTLC. Manipulation of the mobile phase was necessary since the mobile phase used with conventional layers did not separate PG from phosphatidylethanolamine (PE) when used with HPTLC.

MATERIALS AND METHODS

Thin layer plates, LK5 (silica gel) (20 x 20 cm) and LHPK (HPTLC) (10 x 10 cm), were obtained from Whatman, Inc., Clifton, NJ. These were washed by development to the top of the layer using the mobile phase. The layers were then heated in an oven at 150 C for 10 min in order to reactivate. All solvents were of analytical grade distilled in glass.

The samples, 200 ng each, were applied to the pre-absorbant layer with Drummond Microcaps. The chromatograms were developed in unlined tanks which were allowed to saturate with mobile phase for 10 min before the plates were placed in them.

For visualization, the chromatograms were allowed to dry in air until the solvent had evaporated, then dried in an oven at 180 C for 5 min for complete evaporation of solvent, particularly the triethylamine. The chromatograms are then sprayed with a solution of 3% cupric acetate in 8% phosphoric acid (4) until completely wet. Charring was accomplished by heating in an oven at 180 C for 10 min.

After charring, the chromatograms were scanned on a Kontes Chromaflex Densitometer K-495000 (Kontes, Vineland, NJ) using the transmission mode and visible light.

The phospholipids were all obtained from Avanti Biochemicals, Birmingham, Alabama, and included the following: sphingomyelin (S), phosphatidylcholine (L), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). These were dissolved in 10% methanol in chloroform to give a concentration of 100 ng/μl. Two to 5 μl was applied to the starting point of the chromatogram.

RESULTS AND DISCUSSION

The mobile phases listed in Table I all give separation of the six phospholipids, since they all are based on the method originally published (3). The modifications served the purpose of obtaining wider separation in most cases of the lipids. It should be noted that substitution of triethylamine for ethyl acetate and the use of smaller proportions of methanol or aqueous phase resulted in two-fold better separation between PE and PG (see Table I). Figure I shows the separation of the phospholipids from an extract of amniotic fluid prepared by the Gluck method (5) using system B. The separation of the phospholipids on the HPTLC layers using system A resulted in PG and PE moving together. However, using mobile phase B containing triethylamine, the results on the

TABLE I

Mobility of Phospholipids in Various Systems[a]

Mobile Phase Phospholipid	Conventional		HPTLC					
	A	B	A	B	C	D	E	F
S	0.29	0.19	0.30	0.17	0.14	0.18	0.16	0.13
L	0.35	0.26	0.35	0.23	0.20	0.23	0.21	0.17
PS	0.41	0.40	0.46	0.37	0.31	0.36	0.32	0.29
PI	0.48	0.49	0.52	0.46	0.36	0.43	0.38	0.44
PE	0.59	0.55	0.64	0.51	0.42	0.48	0.45	0.57
PG	0.65	0.70	0.64	0.71	0.60	0.66	0.62	0.68

Mobile Phase (in ml)

	A	B	C	D	E	F
Chloroform	25	30	30	30	30	30
Methanol	13	9	9	9		
1-Propanol	25					
2-Propanol		25	25	27		
Ethanol					34	34
Potassium Chloride 0.25%	9	6				
Water			7	8	7	8
Ethyl Acetate	25					
Triethylamine		18	25	25	35	35

[a]Mobility expressed as Rf.

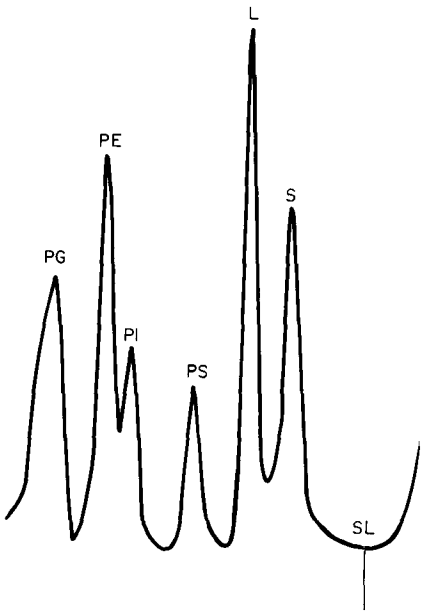

FIG. 1. The phospholipids separated from a term amniotic fluid using system B on a LK5 conventional TLC plate, as described in the text. (SL = Starting Line) See text for key to abbreviations. The amount represents 0.1 ml of amniotic fluid.

HPTLC layers were the same as those obtained on the 20 x 20 cm conventional LK5 layers.

The advantage of using HPTLC lies in faster development (35 min vs. 100 min), as well as the smaller sample required. The HPTLC can be scanned by densitometry with equivalent results. This indicates that use of the principles of solvent selectivity in thin layer chromatography (triethylamine has the same solvent strength as ethyl acetate, but different selectivity) can result in effective change in separation characteristics and improve the quantitative capabilities (6). It should be noted that the R_f values were generally the same on both the conventional TLC and HPTLC, when the same mobile phase is used.

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COMMUNICATIONS

Desaturation of Isomeric *cis* 18:1 Acids

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ABSTRACT

The desaturation of positional *cis* 18:1 isomers (Δ4 through Δ11) was studied, using essential fatty acid deficient rat liver microsomes. The *cis* Δ4, Δ5, Δ6 and Δ7 isomers were not desaturated. The *cis* Δ10 and Δ11 isomers were desaturated at a very low rate. The maximum desaturation was obtained for Δ8 and Δ9 isomers. The *cis* Δ8 and Δ11 isomers were desaturated by Δ5 desaturase; the *cis* Δ9 isomer was desaturated by Δ6 desaturase; and the *cis* Δ10 isomer was desaturated to Δ7,10 and 5,10-18:2 acids.

INTRODUCTION

In the study of the effect of double bond position in fatty acids upon biochemical phenomena, each biochemical reaction or biological response has shown a unique pattern of response relative to the position of the double bonds (1-7). Each biological system was unique in discriminating between locations of *cis* double bonds, and in many cases the displacement of the double bond system one carbon atom along the chain caused drastic difference in acceptability of the fatty acid as substrate.

The hydrolysis by pancreatic lipase of a series of triglycerides each containing one positional isomer of *cis* 18:1 revealed that when the double bond was near the carboxyl group, pancreatic lipase was inhibited (7). The *cis* 18:1 isomers were incorporated at different rates in the cholesteryl ester (CE), triglyceride (TG) and phospholipid (PL) fractions by rat liver mitochondria, and at different positions of these TG and PL fractions according to their double bond position (8). The *cis* 6-18:1 acid was more extensively metabolized than the *cis* 9-18:1 acid in Novikoff hepatoma cells (9). We investigated the desaturation of the isomeric *cis* 18:1 acids by liver microsomes from rats deficient in essential fatty acids (EFA) to see how the position of the double bond affects the rate as well as the site of desaturation of these isomers.

MATERIALS AND METHODS

The labeled positional isomers of *cis* 18:1 acids were prepared in this laboratory. Some of the unlabeled isomers were obtained from Dr. F.D. Gunstone. The purity of the labeled and unlabeled isomers was checked by thin layer chromatography (TLC) and gas chromatography (GC). The purity of all the labeled and unlabeled acids was more than 90%. The double bond position was determined by ozonolysis of the methyl esters and reduction of the ozozonides as described by Privett and Nickell (10). The chain length of the labeled aldehyde ester, which indicates the position of the double bond, was determined by preparative GC using unlabeled carriers of aldehyde esters with chain lengths from C3 to C17.

The optimal concentrations of the substrate, microsomal protein and the time of incubation used for the desaturation of oleic acid were used as reported in a previous study (11).

Each substrate acid was incubated in a concentration of 120 nmoles per ml as Na salt-complex of bovine serum albumin. The substrate contained an amount of 1-^{14}C labeled acid equivalent to 0.1 μCi. Each incubation in 1 ml of a 0.15 M KCl-0.25 M sucrose solution contained in μmoles: ATP, 5; CoA, 0.25; NADH, 1.0; magnesium chloride, 5; glutathione, 1.5; NaF, 45: nicotinamide, 0.5; phosphate buffer (pH 7.0), 100; and 2 mg protein of a microsomal suspension. The microsomes were separated according to the procedure of Marcel et al. (12) by centrifugation at 105,000 x g for 2 hr. Microsomes from EFA deficient rats were used because desaturase activity has been shown to increase in EFA deficiency (12). After 20 min incubation in O_2 at 37 C, the reaction was stopped by adding 1 ml of 5% HCl in methanol, and the lipids were extracted with chloroform/methanol (2:1). The lipids of the extract were dried under nitrogen and transesterified with 10% HCl in methanol at 80 C for 2 hr. The extracts were evaporated to dryness under nitrogen, petroleum ether and unlabeled carriers of 18:0, 18:1 and 18:2 methyl esters were added, and the esters were

separated on 10% $AgNO_3$ TLC plates with petroleum ether (40-60 C)/diethyl ether (85:15, v/v). The quantitative distribution of the radioactivity between the substrate and product as well as the percent conversion and nmoles of product/mg protein/min were calculated as described before (13). The recovery of the radioactivity was more than 90%.

The structure of the dienoic acid esters isolated by TLC was identified by using preparative radio gas chromatography. All the labeled diene esters were found in the fraction corresponding to the 18:2 methyl ester peak. The double bond position was determined as described by Privett and Nickell (10), by partial reduction of the dienoic acids with hydrazine hydrate in methanol to give a mixture of monoenoic and saturated fatty acids. The extent of the reaction was monitored using gas chromatography. When the dienoic esters had just disappeared, the mixture was separated into saturated and monoenoic esters by $AgNO_3$ TLC, using the solvent system of light petroleum ether/diethyl ether (95:5, v/v). The isolated monoenes were ozonized, and the ozonides were reduced to aldehyde and aldehyde esters by Lindlar's catalyst (10). The chain length of the labeled aldehyde esters was identified by preparative GC using unlabeled carriers of aldehyde esters with chain lengths from C3 to C17.

RESULTS AND DISCUSSION

Table I shows that the *cis* 18:1 isomers with the double bond at carbons 4 through 7 were not desaturated at measurable rates by EFA deficient rat liver microsomes. Their percent conversion to 18:2 ranged from 0.48% for Δ4 to 0.76% for Δ6 18:1 acids. The maximum conversion to 18:2 was obtained for *cis* Δ8 and Δ9 isomers which gave 3.4 and 3.9%, respectively. The rate of desaturation of oleic acid to 6,9-octadecadienoate was 0.118 nmoles/min/mg protein, which is comparable to that obtained by Castuma et al. (14) (0.128 ± 0.002 nmoles/mg protein/min). The *cis* Δ10 and Δ11 isomers gave ca. 1.5% conversion to the 18:2 acid.

These results show that the *cis* 18:1 acids with double bond position between carbons 4 and 11 from the carboxyl group are poor substrates for the desaturation, except the *cis* Δ8 and Δ9 isomers were desaturated at relatively higher rates to the 18:2 acid. The *cis* Δ12 18:1 isomer was not desaturated by rat liver microsomes as reported by Gurr et al. (15).

The data in the present study show that the position of the double bond in a *cis* 18:1 acid is a determinant factor for the desaturase which acts on it. The *cis* Δ9 18:1 acid was desaturated by Δ6 desaturase, in agreement with the previous reports (16-18). The site of desaturation was similar for eicosa-9,12,15-trienoic, eicosa-9,12-dienoic (19), heptadeca-9,12-dienoic (20), and hexadeca-9-enoic (18) acids which have different chain lengths and number of double bonds. All of them have the first double bond at the 9-position from the carboxyl group. The *cis* Δ8 and Δ11-18:1 isomers were desaturated by Δ5 desaturase as are eicosa-8,11-dienoic (14), eicosa-8,11,14-trienoic (18), eicosa-11-enoic and eicosa-11,14-dienoic (21) acids, which also have the first double bond at the 8- or 11-positions from the carboxyl group. The desaturation of the *cis* Δ11 18:1 isomers to 5,11-18:2 acid (not to 8,11) confirms the absence of Δ8 desaturase from the rat liver microsomes as reported by Ullman and Sprecher (21).

The *cis* Δ10 18:1 isomers gave 1.57% conversion to the 18:2 acid with ca. 70% (1.1% conversion) of the double bonds at Δ7,10 and

TABLE I

Desaturation of *cis*-18:1 Isomers to 18:2 by EFA Deficient Rat Liver Microsomes Expressed as Percent Conversion and nmoles of 18:2 Produced/mg Protein/min. Values are Means ± SD, n=24

cis-Isomers	% Conversion	nmoles of 18:2/ min/mg protein	Position of double bond of 18:2	Desaturase
cis-Δ4-18:1	0.48 ± 0.47	0.014 ± 0.014		
Δ5	0.47 ± 0.9	0.014 ± 0.027		
Δ6	0.76 ± 0.72	0.023 ± 0.022		
Δ7	0.58 ± 0.32	0.017 ± 0.009		
Δ8	3.44 ± 0.69	0.103 ± 0.021	Δ5,Δ8	Δ5
Δ9	3.92 ± 0.32	0.118 ± 0.009	Δ6,Δ9	Δ6
Δ10	1.57 ± 0.34	0.047 ± 0.010	Δ5,Δ10 + Δ7,Δ10	Δ5 + Δ7
Δ11	1.50 ± 0.25	0.045 ± 0.007	Δ5,Δ11	Δ5

30% (0.47% conversion) as the Δ5,10 18:2 acid. Bernett and Sprecher (18) found that the eicosa-10,13-dienoic acid produced through the palmitoleate sequence was converted in vitro by rat liver microsomes to 7,10,13-20:3 at a very slow rate of only 0.8 nmoles in the 3 min incubation period. The rate of desaturation of *cis* Δ10 18:1 isomer to 7,10-18:2 was only ca. 0.05 nmole/min/mg protein which is lower than that reported for 10,13-20:2 acid (18).

The most abundant *cis* 18:1 isomers in the hydrogenated soybean oil were reported to be the *cis* Δ9 18:1 (76%), and *cis* Δ11 18:1 (10%) (22). The *cis* 18:1 isomers which were found in the present study not to be desaturated by rat liver microsomes (Δ4 → Δ7) were present in less than 1% of the total *cis* 18:1 acids (22). The major *cis*-18:1 acids present in hydrogenated oils are thus potentially metabolizable to *cis,cis*-18:2 acids which, in turn, may be converted to higher polyunsaturated acids and prostaglandin-like compounds.

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Identification of Chondrillasterol in Two *Cucurbitaceae* Seed Oils by Proton Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

The absolute configuration at C-24 of C-24 epimeric 24-ethyl-5α-cholesta-7,E-22-dien-3β-ols I-IV previously isolated from tea seed oil, shea fat, and gourd and sponge cucumber seed oils, respectively, was studied by proton nuclear magnetic resonance spectroscopy. The results showed that the sterols I and II are identical with spinasterol (24S/α-ethyl group), whereas the sterols III and IV are identified as its 24R/β-epimer, chondrillasterol. This study has thus for the first time properly documented the presence in tracheophytes of a 24β-ethylsterol in which $\Delta^{25(27)}$-bond is reduced.

INTRODUCTION

In previous studies (1,2), we isolated C_{29} $\Delta^{7,22}$-sterols I-IV from tea seed oil from *Thea sinensis* L., shea fat from the kernels of *Butyrospermum parkii*, gourd seed oil from *Lagenaria siceraria* S., and sponge cucumber seed oil from *Luffa cylindrica* R., respectively, and determined that their structures are identical with spinasterol [(24S)-24-ethyl-5α-cholesta-7,E-22-dien-3β-ol]. The determination was based essentially on melting points (mp), gas liquid chromatography (GLC), and infrared (IR), mass (MS) and 60 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic analyses and also on literature data (3,4) on higher plant sterols. However, these methods were usually unable to distinguish between epimeric sterols differing only in the absolute configuration at C-24 alkyl groups. In this study, the configuration at C-24 was symbolized by combined use of the (R,S)- and (α,β) nomenclatures for the sake of ready comparison. Recent studies have demonstrated that the C-24 epimeric sterols can be differentiated from each other by 220 or 270 MHz high resolution ^{1}H-NMR (5-7). In addition, the use of a lanthanide shift reagent (LSR) such as a tris[1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-octane-4,6-dionato]ytterbium, $Yb(fod)_3$, in ^{1}H-NMR has provided further excellent results, thus permitting the reliable determination of the configuration at C-24 of an unknown sterol (8).

To determine the absolute configuration at C-24 of the C_{29} $\Delta^{7,22}$-sterols I-IV isolated from the four kinds of vegetable oils and fats, we undertook a re-examination of the sterols by ^{1}H-NMR. Surprisingly, the sterols III (from gourd) and IV (from sponge cucumber) were identified as C-24 epimer of spinasterol, i.e., chondrillasterol [(24R)-24-ethyl-5α-cholesta-7,E-22-dien-3β-ol], which is unusual in higher plants and has hitherto been found only in several species of green algae (*Chlorophyta*) (9).

TABLE I

270 MHz ^{1}H-NMR Data for Methyl Protons of C_{29} $\Delta^{7,22}$-Sterols[a]

Methyl protons	Chemical shifts (δ-ppm)			
	Sterol I (from tea seed oil)	Sterol III (from gourd seed oil)	Spinasterol[b]	Chondrillasterol[b]
C-18 (s)	0.55	0.55	0.55	0.54
C-19 (s)	0.80	0.80	0.81	0.81
C-21 (d)	1.03 (6.6)	1.03 (6.6)	1.03 (6.6)	1.03 (6.5)
C-26 (d)	0.85 (6.4)	0.84 (6.3)	0.85 (6.5)	0.84 (6.5)
C-27 (d)	0.80 (5.7)	0.79 (6.1)	0.80 (ca. 7)	0.79 (ca. 7)
C-29 (t)	0.81 (7.4)	0.82 (7.3)	0.81 (6.5)	0.81 (7.2)

[a]In $CDCl_3$; abbreviations, s=singlet, d=doublet, t=triplet; the values in parentheses refer to the coupling constnats (*J*, in Hz).

[b]Data recorded by Sucrow et al. (7).

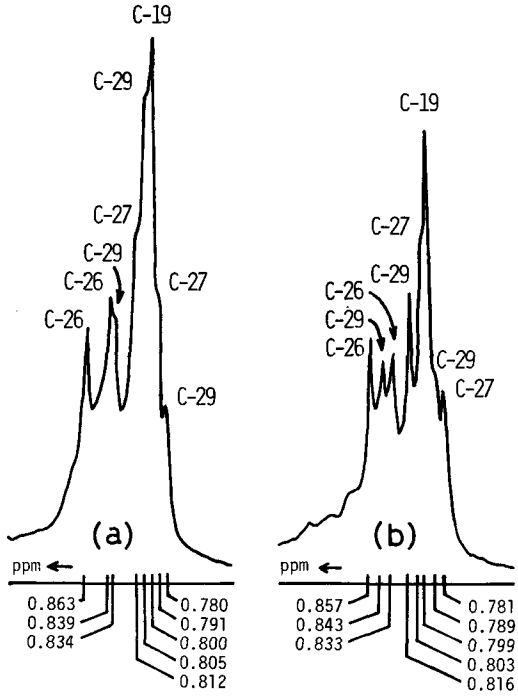

FIG. 1. 270 MHz ^{1}H-NMR spectra of the C-26, C-27, and C-29 methyl proton region of the sterol I (a; from tea seed oil) and III (b; from gourd seed oil).

The present report deals with the occurrence of spinasterol and its 24R/β-epimer, chondrillasterol, in some seed-bearing plants.

MATERIALS AND METHODS

The isolation of the four C_{29} $\Delta^{7,22}$-sterols I-IV examined in this study were reported previously (1,2). An authentic specimen of spinasterol was a gift from Dr. L.J. Goad (University of Liverpool, England); chondrillasterol was a gift from Dr. G.W. Patterson (University of Maryland, MD). The LSR, Yb(fod)$_3$ was available from E. Merck (Darmstadt, Germany).

The ^{1}H-NMR spectra were recorded at 90 and 270 MHz with Hitachi R-22 and Bruker WH 270 NMR spectrometers, respectively, using $CDCl_3$ as solvent and TMS as an internal reference standard (TMS, δ = 0 ppm).

RESULTS AND DISCUSSION

Melting points, GLC retention times and spectral data (IR, MS, and 60 MHz ^{1}H-NMR) of the four C_{29} $\Delta^{7,22}$-sterols I-IV were consistent with the literature values of C-24 epimeric 24-ethyl-5α-cholesta-7,E-22-dien-3β-ol,

TABLE II

Yb(fod)$_3$-Induced Shift Ratios for Side Chain Methyl Protons of C_{29} $\Delta^{7,22}$-Sterols[a]

Methyl protons	Induced shift ratios					
	Sterol I (1.11X10^{-5}M) (from tea seed oil)	Sterol II (1.16X10^{-5}M) (from shea fat)	Sterol III (8.70X10^{-6}M) (from gourd seed oil)	Sterol IV (1.11X10^{-5}M) (from sponge cucumber seed oil)	Spinasterol[b] (6.3X10^{-6}M)	Chondrillasterol[b] (6.7X10^{-6}M)
C-21	1.00	1.00	1.00	1.00	1.00	1.00
C-26	0.38	0.40	0.35	0.35	0.38	0.35
C-27	0.45	0.46	0.43	0.43	0.44	0.42
C-29	0.38	0.40	0.43	0.43	0.38	0.42

[a]Measured at 90 MHz; the values are obtained by the procedures described elsewhere (8).
[b]Authentic samples.

spinasterol (24S/α-ethyl group) and chondrillasterol (24R/β-ethyl group), as previously reported, and were basically similar to one another.

However, several significant differences in the ^{1}H-NMR spectra of the C-26, C-27, and C-29 methyl proton region at ca. δ 0.78-0.86 were observed between the sterol I and III when the spectra were measured at 270 MHz, as shown in Table I and Figure 1. In the sterol III, the doublet due to the C-26 and C-27 methyl protons resonated at higher field (by δ 0.01) than the corresponding protons of the sterol I. Furthermore, the triplet arising from the C-29 methyl proton occurred at lower field (by δ 0.01) in the sterol III than in the sterol I. The remaining parts of the 270 MHz spectra other than the δ 0.78-0.86 region in the two sterols were very similar. These 270 MHz data of the sterols I and III closely resembled those of synthetic C-24 epimers of spinasterol and chondrillasterol, respectively, reported by Sucrow et al. (7), and also strongly suggest that the former has the 24-ethyl group in the α-orientation, while the configuration at C-24 of the latter is β-orientated (4,5).

In order to further confirm the absolute configuration at C-24, Yb(fod)$_3$-induced shift spectra (at 90 MHz) were then measured for the sterols I and III as well as the sterols II and IV by the method previously reported (8). As for the result, there was minor but consistent difference in the Yb(fod)$_3$-induced shifts for the side chain methyl protons between the sterol I-II and III-IV (Table II).

Thus, in the sterols I and II, the signals of the C-27 methyl proton moved somewhat faster on aliquot addition of the LSR than did the C-26 and C-29 methyl signals which are shifted to the same extent. In the sterols III and IV, on the other hand, the C-26 methyl signal moved somewhat slower than did both the C-27 and C-29 methyl protons upon addition of the LSR. The Yb(fod)$_3$-induced shift spectral patterns of the sterols I-II and III-IV were in good agreement with those of authentic spinasterol and chondrillasterol, respectively.

In view of the above findings, it was concluded that the sterols I and II isolated, respectively, from tea seed oil and shea fat are identical with spinasterol, which is usual in higher plants, whereas the corresponding sterols III and IV isolated, respectively, from gourd and sponge cucumber seed oils are identical with chondrillasterol. Therefore, the previous identification of the sterols III and IV as spinasterol should be corrected. The occurrence of chondrillasterol in gourd and sponge cucumber seed oils from *Cucurbitaceae* plants is quite unique, since when a 24-ethyl-$\Delta^{7,22}$-sterol lacking a $\Delta^{25(27)}$-bond has been isolated from tracheophytes, the configuration at C-24 has always been α in the past, though 24-ethylsterols with the $\Delta^{25(27)}$-bond, isolated from some species of tracheophytes, had β-ethyl group at C-24 (3, 4, 10). According to the consideration proposed by Nes et al. (4,11), the biosynthetic pathway of 24β-ethyl-Δ^{22}-sterols is thought to proceed through 24β-ethylsterols with the $\Delta^{25(27)}$-bond as intermediates. Our previous findings that 24-ethyl-$\Delta^{25(27)}$-sterol and its 22,23-dehydro analog together with chondrillasterol, tentatively recognized as spinasterol before the present study, are present in gourd and sponge cucumber seed oils (2) may be an example in support of the above consideration.

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Sterols of Scallop. III. Characterization of Some C-24 Epimeric Sterols by High Resolution (220 MHz) Nuclear Magnetic Resonance Spectroscopy[1]

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ABSTRACT

Alkyl sterols epimeric at C-24 isolated from the Atlantic scallop, *Placopecten magellanicus*, were analyzed by high resolution (220 MHz) nuclear magnetic resonance spectrometry, and their spectra compared with authentic samples. This technique was used to assign absolute stereochemistry in epimeric mixtures of 24 R and S 24-methylcholest-5-en-3β-ol, (22E)-24-methylcholesta-5,22-dien-3β-ol and 24-ethylcholest-5-en-3β-ol. It also allowed a semiquantitative estimate of the R/S isomers present in the mixture.

INTRODUCTION

Marine invertebrates have been shown to contain complex sterol mixtures, and although many new sterols were discovered (1) the analytical procedures available at that time were unable to resolve these complex mixtures completely. More recent investigations into the sterols of marine invertebrates have been reviewed (2). We recently re-examined the sterol mixture of the scallop *Placopecten magellanicus* and isolated several minor sterols as well as several epimeric 24-alkycholesterols (3). High resolution ^1H NMR has been used to evaluate the stereochemistry of sterols epimeric at C-24 (4-6), and we have reported on the utility of ^{13}C NMR for determining stereochemistry at C-24 (7). However, the smaller amounts of samples required and the ready availability of ^1H NMR prompted us to use this method, and to compare the findings with those of the ^{13}C NMR study.

[1]M.S.R.L. Contribution Number 242.

EXPERIMENTAL

The NMR spectra were recorded at 220 MHz on a Varian spectrometer, using $CDCl_3$ solvent and TMS as internal standard. Spectra were recorded at a sweep width of 100 Hz. Peak positions in the spectra are given in Hz and were converted to ppm (Tables I and II) by dividing the values by 220 for the 220 MHz spectra. Epimeric ratios were estimated by planimetry of peak heights.

24-Alkyl sterols were isolated from scallop in a previous study (3). One sample of campe-

TABLE I

Methyl Group Chemical Shifts of 24-Methyl and 24-Methyl-Δ^{22}-Sterols in $CDCl_3$

Sterol	C-24 Config.	C-18[a]	C-19[a]	C-21[b]	C-26[b]	C-27[b]	C-28[b]
Mixture *1a* and *1b* (scallop)	S/β R/α	0.679[c]	1.007	0.919 0.911	0.856 0.852	0.783 0.704	0.775
Mixture *1a* and *1b* (synthetic)	S/β R/α	0.682	1.012	0.923 0.915	0.860	0.785 0.814	0.780
Mixture *1a* and *1b* (nutritional biochem.)	S/β R/α	0.685	1.013	0.923 0.916	0.856	0.787 0.808	0.778
Campesterol (appl. sci.) *1b*	R/α	0.687	1.012	0.915	0.857	0.808	0.779
(22E,24ξ)-24-Methylcholesta-5,22-dien-3β-ol *3a* and *3b* (scallop)	S/α R/β	0.698	1.012	1.007 1.015	0.841	0.823	0.915 0.934
(22E,24S)-24-Methylcholesta-5,22-dien-3β-ol *3a* (synthetic)	S/α	0.700	1.013	1.006	0.841	0.823	0.915
22E,24S)-24-Methylcholesta-5,22-dien-3β-ol *3a* (diatom)	S/α	0.699	1.015	1.009	0.842	0.824	0.916

[a]Singlet.

[b]Doublet.

[c]All values given in ppm.

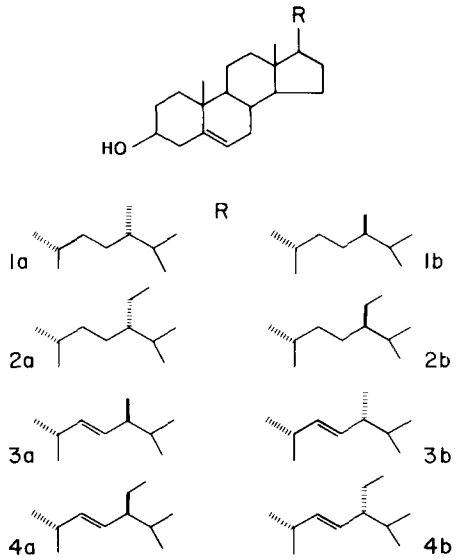
TABLE II

Methyl Group Chemical Shifts of 24-Ethyl and 24-Ethyl-Δ^{22}-Sterols in $CDCl_3$

Sterol	C-24 Config.	C-18	C-19	C-21	C-26	C-27	C-28
Clionasterol *2a*	S/β	0.683[a]	1.011	0.928	0.834	0.814	0.855
Sitosterol *2b*	R/α	0.684	1.011	0.925	0.839	0.815	0.842
Mixture of *2a* and *2b* (scallop)	S/β R/α	0.683	1.006	0.925	0.839	0.816	0.848
Poriferasterol *4b*	R/β	0.699	1.009	1.025	0.845	0.793	0.880 0.812
Mixture of *4a* and *4b* (scallop	S/α R/β	0.701	1.014	1.028	0.848	0.795	0.814
Stigmasterol (24S) *4a*	S/α	0.703	1.012	1.026	0.849	0.799	0.808

[a]All values given in ppm.

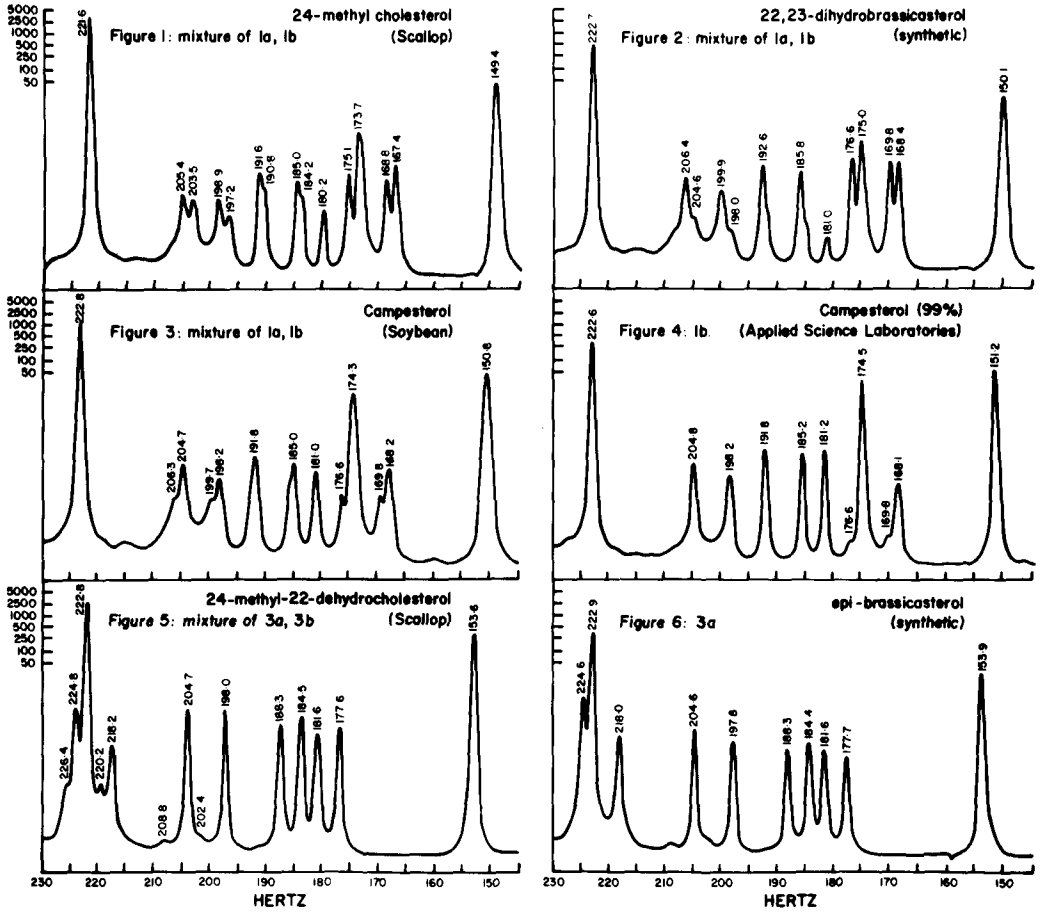
sterol *1b* was purchased from Applied Science Laboratories, State College, PA, while a second sample of "campesterol" *1b* from soybean was obtained from Nutritional Biochemicals, Cleveland, OH, and contained approximately one-third 22, 23-dihydrobrassicasterol *1a*. Clionsterol (24S)-24-ethylcholest-5-en-3β-ol *2a* was isolated from *Nitella* (8) while sitosterol (24R)-24-ethylcholest-5-en-3β-ol *2b* was isolated from soybean. 22-Dehydrocampesterol (22E, 24R)-24-methylcholesta-5,22-dien-3β-ol, *3a*, was isolated from the diatom *Phaeodactylum tricornutum*. Dr. M.J. Thompson (USDA, Beltsville, MD) provided synthetic samples of *3a* (epibrassicasterol) and *1a* (dihydrobrassicasterol), and the latter was shown to contain approximately one-third campesterol *1b*. Stigmasterol (22E,24S)-24-ethycholesta-5,22-dien-3β-ol), *4a*, was purchased from Applied Science Labs, while poriferasterol, the 24R epimer was obtained from *Spirogyra*, a fresh water alga.



RESULTS AND DISCUSSION

(24R)-24-Methylcholest-5-en-3β-ol *1b* and (24S)-24-Methylcholest-5-en-3β-ol *1a*

The methyl group chemical shifts of four samples of 24-methylcholesterol are listed in Table I, and the spectra are illustrated in Figures 1-4; these are scallop sterol (Fig. 1), a synthetic sample of "22,23-dihydrobrassicasterol" (Fig. 2), and campesterol samples from soybean (Nutritional Biochemicals) and Applied Science. Assignments of the methyl group of campesterol (Applied Science, Table I) agree well with published data (4-6), but comparison of the spectrum of the scallop sample shows certain differences (Table I). In the scallop sterol spectrum, there are two pairs of doublets at 0.911 and 0.919 ppm (Table I) assigned to the C-21 methyl resonance of the 24R and 24S epimers, respectively. This difference of ∿2 Hz is also found in the spectra of the other epimeric mixtures of 24-methylcholesterol examined. There are two pairs of doublets at 0.783 and 0.804 ppm assigned to C-27 methyl resonances for the 24S and 24R epimers. The chemical shift difference of 0.02 ppm (4.4 Hz) is constant for the epimeric mixtures examined, and agrees with the observation by Rubinstein et al. (5) that the C-27 methyl resonance is most sensitive to C-24 stereochemistry and is downfield in 24α sterols (e.g., *1b*); it is therefore of diagnostic value in assigning C-24 stereochemistry in saturated 24-methyl sterols. Estimation of peak heights of the C-21 methyls established that the scallop sterol is an approximately equal mixture of the 24R and 24S epimers; the synthetic sample (Fig. 2) is a mixture containing two-thirds *1a*, while the soybean sample (Fig. 3) contains approximately equal amounts of *1a* and *1b*; a small amount of the 24S epimer was detected in campesterol from Applied Science.



FIGS. 1-6. 220 Hz spectra of sterols; sweep width 100 Hz.

(22E,24R)-24-Methylcholesta-5,22-dien-3β-ol *3b* (Brassicasterol), and (22E,24S)-24-Methylcholesta-5, 22-dien-3β-ol *3a* (22-dehydrocampesterol)

Three samples were examined: a diastereoisomeric mixture from the scallop (Fig. 5), a sample of the 24S isomer, 22-dehydrocampesterol (Fig. 7) from the diatom *P. tricornutum*, and a synthetic sample, also with 24S stereochemistry (Fig. 6). The methyl group chemical shifts are summarized in Table I. Inspection of the spectra shows that the 24R and S epimers can be distinguished by the differences in the chemical shift of the C-21 methyl, which is at lower field in the 24R (β) series. The C-28 methyl group is less sensitivie to stereochemistry.

The sensitivity of the C-21 methyl group to stereochemistry in ^{1}H NMR offers an additional diagnostic tool since ^{13}C NMR (7) has shown that the most useful signals for differentiating these epimers are the resonances at C-28 and C-16. Chemical shift differences for the C-21 and C-28 resonances using 100 MHz ^{1}H NMR were also used to assign stereochemistry at C-24 (9) in the diatom *P. tricornutum*.

Estimates that the scallop sample contains about two-thirds 24S epimer, 3a(∿65%) by integration of the C-21 methyl resonance, though not as precise, are in good agreement with values from ^{13}C NMR (7). These new data confirm the earlier suggestion that (22E,24ξ)-24-methylcholesta-5,22-dien-3β-ol from scallop might be a mixture of the C-24 epimers from which brassicasterol was isolated (10). Synthetic *3a* (Fig. 6) is included for comparison and is very similar to the diatom sterol (Fig. 7) whose assignment (9) as 24S is confirmed. The Japanese scallop *Patinopecten yessoensis* has been shown to contain *3a* (11), but these workers did not mention whether it occurred along with lesser amounts of *3b*, as in *P. magellanicus*.

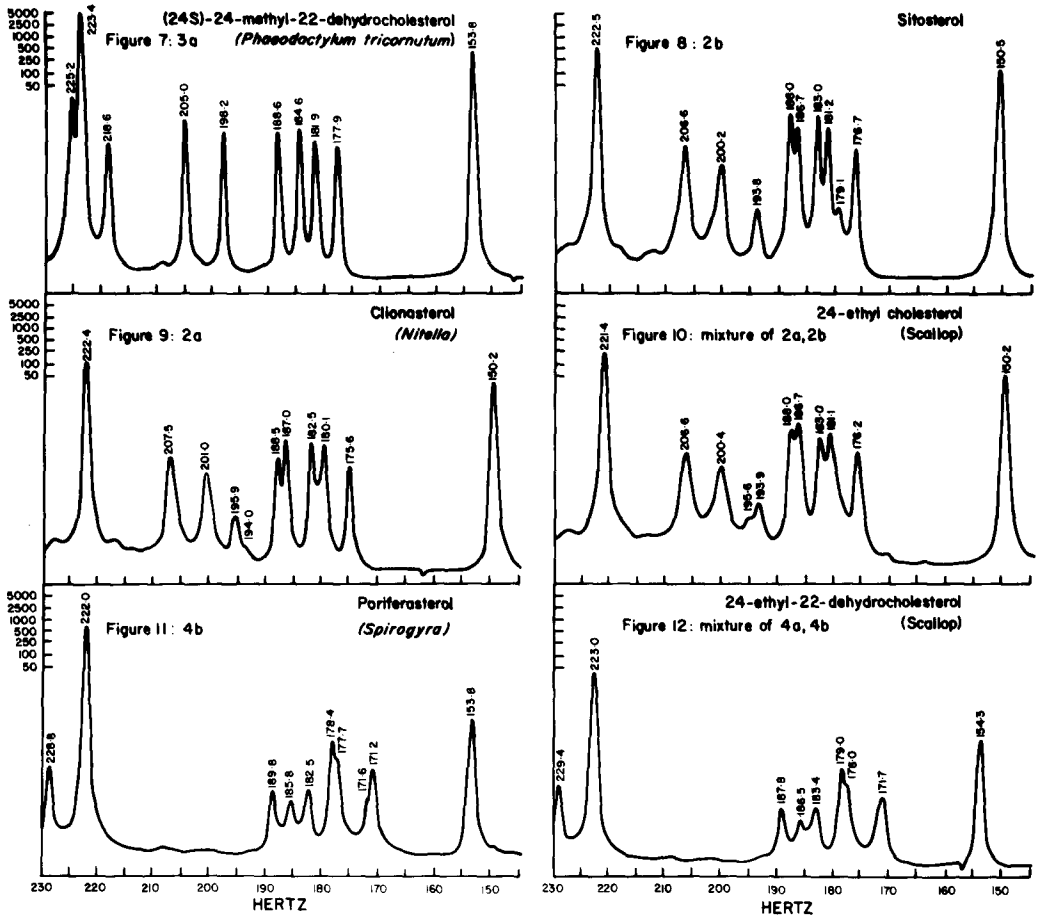
(24R)-24-Ethylcholest-5-en-3β-ol *2b* and (24S)-24-Ethylcholest-5-en-3β-ol *2a*

Three samples were examined: sitosterol *2b* from soybean (Fig. 8), clionasterol *2a* (Fig. 9) isolated as the acetate from Nitella (8), and a mixture isolated from scallop (Fig. 10). Previous studies (5,6) have realized the difficulty of differentiating C-24 diastereoisomers of C_{29} sterols, although differentiation by ^{13}C NMR has already been pointed out (7). The C-29 methyl resonance of the 24S epimer 2a is more deshielded than that in the 24R isomer *2b*, and offers the only major difference. It is, however, necessary to obtain spectra of both diastereoisomers; and comparison shows that the scallop sterol is clearly a mixture with the 24R epimer the major sterol (∿65%). While precise determinations of epimeric ratios are not possible by ^{1}H NMR as with ^{13} C NMR (67%R:33%S) (7), the former provided a quite good estimation of the relative amounts in this instance. When pure samples are available, the acetate of *2b* m.p. 122C can be distinguished from 2a acetate m.p. 143 C (4).

(22E,24R)-24-Ethylcholesta-5,22-dien-3β-ol-*4b* and (22E,24S)-24-Ethylcholesta-5,22-dien-3β-ol-*4a*

Three samples were examined: pure 24R (poriferasterol, Fig. 11), 24S (stigmasterol, Fig. 13), and an epimeric mixture from scallop (Fig. 12). Methyl group chemical resonances are recorded in Table II. Comparison of these spectra shows that the scallop sterol contains predominantly the 24R epimer *4b*. As for mixtures of compounds *2a* and *2b*, ^{1}H NMR does not easily differentiate between the R and S epimers in a mixture of *4a* and *4b*, but estimation is still possible with ^{13}C NMR (7).

In summary, 220 MHz ^{1}H NMR was used to assign configuration at C-24 of mixtures of 24 alkyl sterols isolated from the scallop, by comparison with authentic samples, and to give a qualitative estimate of the R/S ratios.



FIGS. 7-12. 220 Hz spectra of sterols; sweep width 100 Hz.

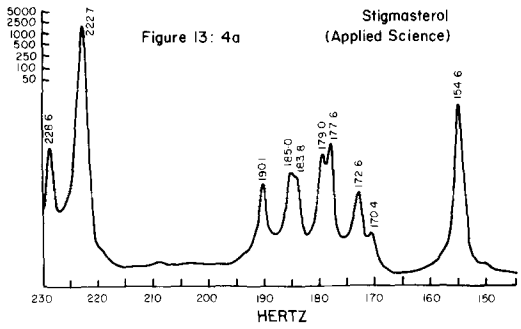


FIG. 13. 220 Hz spectra of sterols; sweep width 100 Hz.

Previous studies (4-6,12) have also demonstrated the functional utility of this method, and have noted its limitations. It has been pointed out that in higher plants C_{28} and C_{29} sterols (24α) with saturated side chains are more common than those with 22-double bonds, whereas in algae the reverse is true (13). In scallop, which apparently obtains sterols from many sources, 24α sterols (e.g., *1b,2b*) predominate when the side chain is saturated, while in the 22-dehydrosterols, scallop contains mixtures of both 24α (e.g., *3a*) and 24β (e.g., *4b*) epimers (Tables I-II and Figs. 1-3).

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Different Metabolism of Saturated and Unsaturated Long Chain Plasma Free Fatty Acids by Intestinal Mucosa of Rats

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ABSTRACT

During fat absorption, unsaturated long chain fatty acids are esterified at a higher rate than saturated fatty acids of similar chain length. This phenomenon has been attributed to differences in the binding affinity of fatty acids to a cytosolic fatty acid-binding protein. As intestinal mucosa utilizes plasma free fatty acids as well, we investigated whether long chain *plasma* free fatty acids of different degree of saturation are metabolized also at different rates. ^{3}H-Palmitic and ^{14}C-linoleic acid complexed to rat serum were injected rapidly into a tail vein of fasting rats. One, 2 and 4 min later there was no difference between ^{3}H and ^{14}C-radioactivity in intestinal mucosa, suggesting equal initial uptake of the two labeled fatty acids from plasma. Despite their equal uptake, the incorporation of the isotopes into ester lipids was significantly different, however: at 2 min, 53.1 ± 3.9% of ^{3}H and 73.8 ± 4.6% of ^{14}C were recovered in ester lipids. Phospholipids and triglycerides accounted for most of the mucosal ^{3}H and ^{14}C. At 4 min, a similar distribution of isotopes in intestinal mucosal metabolites was found. These data show that despite equal initial uptake by intestinal mucosa unsaturated long chain fatty acids taken up from plasma are esterified to a higher and oxidized to a lower extent than saturated plasma free fatty acids. Unsaturated plasma free fatty acids, therefore, may provide a more important source of fatty acids for endogenous intestinal lipoprotein lipids than saturated plasma free fatty acids. It is speculated that the fatty acid binding protein might be operative not only in the intracellular transport and metabolism of luminal fatty acids but of plasma free fatty acids as well.

INTRODUCTION

Fatty acid metabolism has been studied already in great detail in many tissues. The interest in studies of fatty acid metabolism in intestinal mucosa is borne by the unique duality of the fatty acid supply to this tissue, which takes up fatty acids not only from the intestinal lumen, but from the plasma pool as well (1-5).

Long chain fatty acids absorbed by intestinal mucosa from the lumen are mainly esterified and incorporated into lipoproteins, which then are secreted into the intestinal lymph (6).

Recently it has been demonstrated that unsaturated long chain fatty acids taken up from the intestinal lumen are esterified at a higher rate than saturated fatty acids of similar chain length (7). This difference in the esterification rate has been attributed to the presence of a protein of low molecular weight in the cytosol of absorptive epithelial cells (7). This protein, designated fatty acid binding protein (FABP), binds fatty acids and participates in the translocation of fatty acids from the microvillous membrane to the smooth endoplasmic reticulum (7-9) where fatty acids are activated and esterified. As the binding affinity of fatty acid binding protein is higher for unsaturated than for saturated long chain fatty acids, the higher esterification rate of absorbed long chain unsaturated fatty acids has been explained on this account (7-9).

From studies undertaken by Gangl in rats (4), and more recently also in man (5), we know that intestinal mucosa not only utilizes luminal fatty acids but plasma free fatty acids as well. We, therefore, investigated whether long chain fatty acids of different degree of saturation taken up from the plasma pool are metabolized also at different rates by intestinal mucosa. The results of these studies have been presented in part at the 10th International Congress of Gastroenterology in Budapest, Hungary, 1976, and at the 33rd Meeting of the Deutsche Gesellschaft für Verdauungs- und Stoffwechselkrankheiten in Hamburg, Germany, 1978, and have been published in abstract form (10,11).

MATERIALS AND METHODS

Spectrum of Plasma Free Fatty Acids of Animals Studied

All studies were performed in unanaesthetized male albino rats of 300-400 g body weight. In a pilot study, the spectrum of the plasma free fatty acids was analyzed by gas chromatography in 12 rats fasted for 24 hr. Lipids were extracted from 0.5 ml of serum according to Folch et al. (12) and separated

into individual lipid classes by thin layer chromatography (TLC), using TLC plates precoated with silica gel from E. Merck, Darmstadt, Germany, which were "washed" prior to their use by allowing them to develop overnight in chloroform/methanol, 49:1 (13). Plates were developed in a mixture of petroleum ether/diethyl ether/acetic acid (90:15:1.5), sprayed with Rhodamin 6 G, and individual lipid classes were identified by comparison with appropriate lipid standards. The fatty acid zone was eluted from silica gel by 3 extractions with chloroform/heptane/methanol (280:210:10); fatty acids were methylated with boron fluoride-methanol following the method of Morrison and Smith (14) and separated into individual fatty acids in a gas chromatograph (Fractovap 2400 by Carlo Erba) using a glass column of 2 m length and 4 mm ID, packed with 3% Hi Eff 8BP on Chromosorb 6 HP 100/120 mesh. Nitrogen at a flow of 25 ml/min was used as the carrier gas.

Radio-Isotopes Used

9,10-^{3}H-Palmitic acid (500 m Ci/mmol) and 1-^{14}C-linoleic acid (60 m Ci/mmol) radiochemically > 97% pure were bought from the Radiochemical Centre, Amersham. At the start of each experiment, a trace amount of ^{3}H-palmitic acid and of ^{14}C-linoleic acid was complexed with 2.5 ml of fresh serum from donor rats which then was diluted 1:1 with 0.9% saline as described earlier (4).

Experimental Procedure

One ml of the ^{3}H-, ^{14}C-fatty acid-rat serum complex was injected acutely into a tail vein of conscious rats after a fasting period of 24 hr. One, 2 and 4 min later rats were decapitated by means of a guillotine, the blood was collected and the serum was separated by centrifugation. The small bowel was excised immediately and flushed with 50 ml of ice-cold saline. From the proximal half of the small bowel (jejunum), the mucosa was isolated by squeezing on a chilled glass plate. The extruded mucosa was weighed and homogenized instantly in a Teflon-glass homogenizer in 3 volumes of methanol. Lipids were extracted from aliquots of homogenates and serum and separated into individual lipid classes by TLC as described above. The triglyceride and fatty acid zones were eluted from the silica gel by 3 extractions with chloroform/heptane/methanol, 280:210:10, and the eluates were used for quantitative biochemical determination of triglycerides (15) and fatty acids (13).

TABLE I

Percentage Composition of Plasma Free Fatty Acids (FFA) of 12 Fasting Rats[a]

Fatty acid		% of "total" FFA (mean ± 1 SE)
Myristic acid,	14:0	4.8 ± 0.8
Myristoleic acid,	14:1	1.6 ± 0.4
Palmitic acid,	16:0	31.2 ± 0.4
Palmitoleic acid,	16:1	10.7 ± 0.7
Stearic acid,	18:0	6.2 ± 0.3
Oleic acid,	18:1	19.8 ± 0.8
Linoleic acid,	18:2	17.8 ± 1.2
Linolenic acid,	18:3	1.6 ± 0.2
Arachidic acid,	20:0	0.1 ± 0.1
Arachidonic acid,	20:4	6.2 ± 0.6
Unsaturated "total"		57.7 ± 0.9
Saturated "total"		42.3 ± 0.9

[a]Rats were fasted for 24 hr, decapitated, and the blood was collected. Lipids were extracted from serum, free fatty acids were separated by TLC, and individual free fatty acids were determined gas chromatographically as described in Methods.

Radioassays

Homogenates and whole serum were assayed for ^{3}H and ^{14}C radioactivity in Liquifluor toluene solution (New England Nuclear) containing 10% Biosolv (Beckman) in a Beckman liquid scintillation counter (model LS 3150 T). For lipid extracts, Biosolv was not added. Quenching was corrected for by an automatic external standard.

Water soluble radioactivity was taken as a parameter of fatty acid oxidation and calculated as the difference between radioactivity in the whole homogenate and the lipid extract (4).

RESULTS

The percentage distribution of individual long chain fatty acids in the serum of the experimental animals is shown in Table I. Of fatty acids of a chain length from C14 to C20, 57.7% were unsaturated and 42.3% were saturated. As the main representative of saturated serum free fatty acids, we found palmitic acid (31.2%); among the unsaturated serum free fatty acids, oleic acid (19.8%) and linoleic acid (17.8%) prevailed.

Following the i.v. pulse injection of a trace amount of ^{3}H-palmitic acid as a marker of saturated and of ^{14}C-linoleic acid as a marker of unsaturated long chain serum free fatty acids, the radioactivity of intestinal mucosa was highest at 2 min and amounted to 0.43% of injected ^{14}C and to 0.44% of injected ^{3}H per gram wet mucosa (Fig. 1). There was no dif-

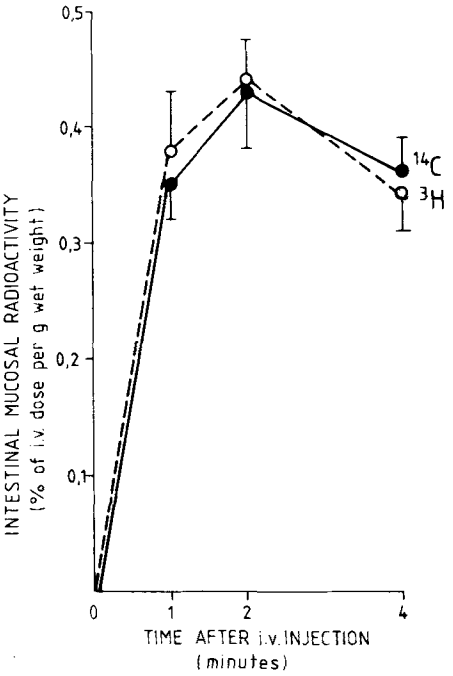

FIG. 1. Uptake of ^{3}H-palmitic and ^{14}C-linoleic acid by intestinal mucosa from plasma. ^{3}H-palmitic and ^{14}C-linoleic acid, complexed to rat serum, were injected acutely i.v., and ^{3}H and ^{14}C was measured in intestinal mucosal homogenates (see Methods) at the times indicated. Each point represents the mean of at least 10 experiments, the vertical lines indicate the Standard Error.

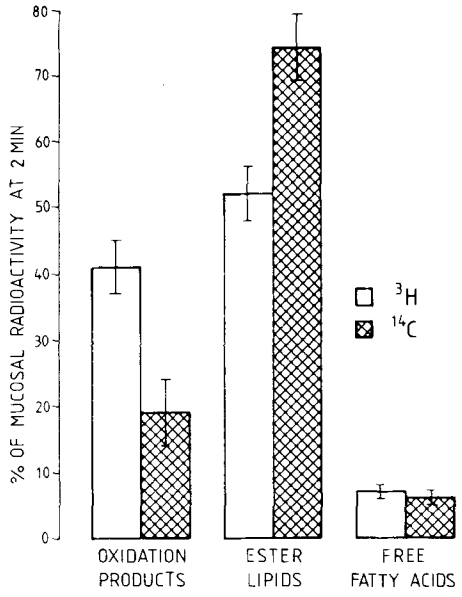

FIG. 2. Incorporation of ^{3}H and ^{14}C into intestinal mucosal metabolites 2 min after acute simultaneous i.v. injection of ^{3}H-palmitic and ^{14}C-linoleic acid complexed to rat serum. Bars represent means of 12 experiments; vertical lines indicate the Standard Error.

ference between ^{3}H and ^{14}C radioactivity of intestinal mucosa at 1, 2 and 4 min. This means that the two labeled fatty acids were taken up by intestinal mucosa to a similar extent. In order to obtain information about the intracellular metabolic fate of the two fatty acids injected i.v., we determined the distribution of the isotopes in intestinal metabolites at 2 and 4 min after the i.v. injection.

Figure 2 shows the ^{3}H and ^{14}C radioactivity in water soluble oxidation products, ester lipids and free fatty acids of intestinal mucosa 2 min after simultaneous i.v. injection of ^{3}H-palmitic and ^{14}C-linoleic acid. The radioactivity in these metabolic products is expressed as a percentage of the mucosal radioactivity of each isotope at 2 min. At this time, there was a significantly higher percentage (38.7%) of ^{14}C, the label of linoleic acid, in ester lipids, than of ^{3}H, the label of palmitic acid ($p < 0.01$; paired t-test). Alternatively, water soluble oxidation products contained significantly less ^{14}C than ^{3}H ($p < 0.01$). By contrast, radioactivities in free fatty acids of intestinal mucosa were equal. A similar pattern of results was seen also at 4 min after i.v. injection (Table II). These findings demonstrate that during the first 4 min after i.v. injection a significantly greater fraction of the unsaturated (linoleic) fatty acid was esterified and a smaller fraction was oxidized, despite equal initial uptake of linoleic and palmitic acid by intestinal mucosa. Determination of ^{14}C and ^{3}H in individual lipid classes of intestinal mucosa 2 min after i.v. injection of the labeled fatty acids showed that phospholipids and triglycerides accounted for the majority of the ester lipid radioactivity. ^{14}C, the marker of linoleic acid, was significantly higher in phospholipids and triglycerides when compared with ^{3}H, the marker of palmitic acid (Fig. 3).

The concentrations of free fatty acids and of triglycerides in serum and in intestinal mucosa are shown in Table III. There are no major differences between rats sacrificed at different points in time.

DISCUSSION

The results of these studies confirm the earlier finding of others (1-3) and of one of us (A.G.) (4,5) that intestinal mucosa takes up fatty acids from the plasma pool. For the first time, however, our data show that in vivo the extent of initial uptake of a saturated long

TABLE II

Incorporation of ^{3}H and ^{14}C into Ester Lipids and into Free Fatty Acids of Intestinal Mucosa at 2 and 4 Min after the Injection of ^{3}H-Palmitic and ^{14}C-Linoleic Acid i.v.[a]

	Radioactivity (% of respective mucosal radioactivity)			
	in ester lipids		in free fatty acids	
	2 min	4 min	2 min	4 min
^{3}H (16:0)	53.1 ± 3.9	60.6 ± 2.8	7.1 ± 1.0	4.6 ± 1.5
^{14}C (18:2)	73.8 ± 4.6	70.4 ± 3.4	6.0 ± 1.1	4.4 ± 1.5
Difference	+ 20.7	+ 9.8	- 1.1	- 0.2
Significance of difference	$p < 0.01$	$p < 0.05$	N.S.	N.S.

[a]^{3}H-palmitic and ^{14}C-linoleic acid complexed to rat serum were injected acutely into a tail vein of fasting rats. Two and 4 min later, rats were decapitated, small bowel mucosa was isolated, and lipids were extracted and separated into individual lipid classes by TLC. ^{3}H and ^{14}C were measured in ester lipids and in free fatty acids (Methods) and expressed as % of total mucosal ^{3}H and ^{14}C-radioactivity, respectively. Data are given as means ± 1 Standard Error; comparison was made by paired t-test.

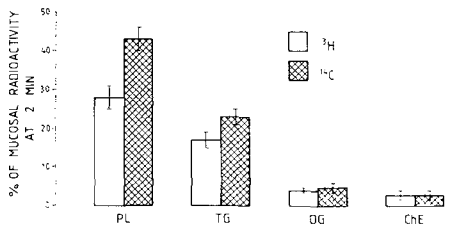

FIG. 3. Incorporation of ^{3}H and ^{14}C into individual ester lipid classes of intestinal mucosa 2 min after acute i.v. injection of ^{3}H-palmitic and ^{14}C-linoleic acid. PL = phospholipids; TG = triglycerides; DG = diglycerides; ChE = cholesteryl esters. Bars represent means of 12 experiments; the vertical lines indicate the Standard Error.

chain fatty acid (palmitic acid) by intestinal mucosa from plasma does not differ from that of an unsaturated long chain fatty acid (linoleic acid), and that despite the equal uptake of the two fatty acids the unsaturated fatty acid is esterified to a higher and oxidized to a lower extent than the saturated fatty acid.

Thus, our studies reveal a difference in intestinal metabolism of plasma free fatty acids of similar chain length but of different degree of saturation. This difference is similar to that described earlier in vitro for fatty acids taken up from the intestinal lumen (7). The underlying cause of this metabolic difference cannot be elucidated conclusively by the data presented. Because of the similarity in the metabolic difference between saturated and unsaturated long chain fatty acids in our studies of the intestinal metabolism of *plasma* free fatty acids with the metabolic difference described earlier for *luminal* fatty acids, it is tempting to speculate that the metabolic difference seen in our studies might be related to the fatty acid binding protein too. This protein exerts a higher binding affinity to unsaturated than to saturated long chain fatty acids, and its main function is thought to be the translocation of long chain fatty acids from the microvillous membrane to the endoplasmic reticulum (8,9). Possibly, the function of this cytosolic protein is not confined to fatty acid absorption but includes the metabolism of long chain *plasma* free fatty acids as well. As plasma free fatty acids approach the absorptive intestinal epithelial cell at its basal pole opposite to the brush border, one only has to assume that fatty acid binding protein is present also in the cytosol of the basal cell pole, and is operative there in a similar way as in the apical cytosol – an assumption which is not unreasonable.

Finally, as plasma linoleic acid was esterified to a higher extent than palmitic acid, it is conceivable that quantitatively unsaturated plasma free fatty acids may provide a more important source of fatty acids for endogenous intestinal lipoprotein lipids than saturated plasma free fatty acids. Studies of additional examples of saturated and unsaturated long chain fatty acids are needed in order to

TABLE III

Concentration of Free Fatty Acids (FFA) and of Triglycerides (TG) in Serum and in Intestinal Mucosa of Rats Sacrificed 1, 2 and 4 min after i.v. Injection of ^{3}H-Palmitic and ^{14}C-Linoleic acid[a]

Time after i.v. injection (min)	Serum (μmol/ml)		Mucosa FFA (μmol/g)
	FFA	TG	
1	0.55 ± 0.02	0.17 ± 0.01	0.79 ± 0.03
2	0.48 ± 0.03	0.16 ± 0.02	0.82 ± 0.14
4	0.57 ± 0.02	0.16 ± 0.02	---

[a]A trace amount of ^{3}H-palmitic and ^{14}C-linoleic acid was injected rapidly into a tail vein of fasting rats. One, 2 and 4 min later rats were decapitated, the blood was collected and the small bowel mucosa was isolated immediately. Lipids were extracted from serum and mucosal homogenates, FFA and TG were isolated by TLC and measured quantitatively as described in Methods. Data are mean ± 1 Standard Error.

establish definitely the validity of this general conclusion, however.

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Turnover of Label from [1- ^{14}C]Linolenic Acid in Phospholipids of Coho Salmon, *Oncorhynchus kisutch*[1]

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ABSTRACT

Juvenile coho salmon were injected intraperitoneally with [1-^{14}C] linolenic acid, and sampled at 24, 120, and 240 hr. Liver, heart, and gill lipids were extracted, analyzed, and halflives of individual liver glycerophospholipids and n-3 fatty acids determined from rates of loss of radioactivity. Incorporation of label into gill was much less than into either heart or liver. Total acyl halflife was shorter for the choline phospholipids than for the ethanolamine phospholipids, as were the halflives of all individual n-3 fatty acids. Eicosapentaenoic acid (20:5n-3) had the shortest halflife in both phospholipids (50-60 hr), while docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (22:6n-3) had much longer halflives. Specific activities of the shorter chain n-3 fatty acids were much greater than the longer, more unsaturated homologs at all times, suggesting possible differences in their mechanisms of incorporation into phospholipids. Diacylglycerol analysis indicated that de novo synthesis could be responsible for the incorporation of only a small portion of the labeled long chain fatty acids found in phospholipids. The fatty acid halflives reported here for salmon are in general agreement with those found previously in mammals.

INTRODUCTION

It is well established that certain species of fish require fatty acids of the n-3 family for normal growth and development. Dietary studies with salmonids have shown that while linolenic acid (18:3n-3) efficiently fulfills the essential fatty acid requirement of these fish (1-3), its ultimate conversion product, docosahexaenoic acid (22:6n-3), also satisfies this requirement and seems to be the fatty acid required at the tissue level (4). Although mammals have been shown to require fatty acids of the n-6 family, acids of the n−3 family are found in large quantities in certain vital tissue lipids. Efforts to deplete n-3 content through dietary manipulation have proved difficult, indicating that these acids perform some as yet undetermined function. Several functions have been attributed to the essential fatty acids and the dynamics of these compounds, i.e., their rates and patterns of incorporation and turnover have been studied both in vivo and in vitro (5-9).

Investigators have found that the level of essential fatty acids in the phospholipids of fish can be altered by diet (2,10) or environmental temperature (11-13) and that fish differ in their capability to desaturate dietary linolenic acid (14). However, little information is available on the incorporation and metabolism of individual n-3 fatty acids in fish. It would seem that detailed studies on this subject could yield valuable information on the function of these molecules in both fish and mammals. This report describes the incorporation of (1-^{14}C) linolenic acid in coho salmon and the turnover of n-3 fatty acids in the major hepatic phospholipids.

MATERIALS AND METHODS

Radiochemicals

[1-^{14}C] Linolenic acid, >99% pure and 50 mCi/mmole, was purchased from Amersham Corp., Arlington Heights, IL. Counting was carried out in a Nuclear-Chicago 720 liquid scintillation counter, using as fluors either toluene-PPO-POPOP (15) or Aquasol (New England Nuclear, Boston, MA).

Animal Trials

Fish used in this study were yearling coho salmon, hatched and reared in circular tanks at the Food Toxicology and Nutrition Laboratory, Corvallis, OR. Fish were fed a semi-purified diet (16) containing 6% salmon oil as lipid source. Water temperature was constant at 12 C. Fish fasted 48 hr, weighing 90 ± 15 g and with noticeable parr marking were injected intraperitoneally with [1-^{14}C] linolenic acid in 4:1 DMSO-0.9% NaCl (0.10 μCi/μl) at a dose of 0.075 μCi/g fish. Three fish were used for each of the following three time periods: 24, 120, and 240 hr. All fish were fasted for the duration of the experiments. The 24 and 120 hr trials were carried out in plexiglass metabolism chambers with recirculating water maintained at 12 ± 0.5 C by a refrigeration-pump unit

[1]Technical Paper No. 5238, Oregon Agricultural Experiment Station, Oregon State University, Corvallis, Oregon 97331.

[2]This material is taken in part from a thesis submitted in partial fulfillment of the requirements for the MS degree in 1978.

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(Lauda K-2/R, Brinkmann Instruments, Burlingame, CA). The sealed chambers were aerated by N_2/O_2 (80:20) and the effluent flow was bubbled through 20% KOH to trap radioactive carbon dioxide expired by the fish. Following termination of the trial, the chamber was acidified with concentrated HCl and the system purged for an additional hour. Ten day trials were carried out in open isolated tanks at the Food Toxicology and Nutrition Laboratory.

Lipid Extraction and Analysis

At various time periods, fish were killed by a blow on the head. Livers, hearts, and gills were excised, perfused with 0.9% NaCl and either extracted immediately or frozen on solid CO_2 and stored at -40 C under nitrogen. Lipid extracts were prepared by the procedure of Folch et al. (17), dried, weighed, and samples taken for liquid scintillation counting. All lipids were stored under nitrogen in benzene at -40 C until analyzed.

Duplicate phosphorus analyses were done according to the method of Bartlett (18), after using two dimensional thin layer chromatography (TLC) (19) to separate individual phospholipids. Additional plates were run to obtain phospholipid samples for counting. Phospholipids were identified by comparison with standards and by specific spray reagents (20). Choline and ethanolamine glycerophospholipids (CGP and EGP) for fatty acid analysis were obtained by one dimensional preparative TLC using chloroform/methanol/water (65:25:4). Diacylglycerols were isolated by TLC with benzene/ethyl acetate/glacial acetic acid (80:20:1) for 13 cm, then rechromatographing in the same direction for 18 cm with hexane/diethyl ether/glacial acetic acid (80:20:1). Cholesteryl esters were isolated using only the second system. Lipid bands were visualized with 2,7-dichlorofluorescein under UV, scraped into ampoules, and transesterified under nitrogen with 4% sulfuric acid in methanol. Methyl esters were extracted with hexane, and samples of both aqueous and hexane fractions were taken for counting.

EGP and CGP were analyzed for plasmalogen content by the procedure of Horrocks (21), and results showed that these compounds were present only in trace amounts.

Fatty acid methyl esters were analyzed using a Varian Aerograph 1200 gas chromatograph (FID), with a 6 ft stainless steel column packed with 15% ethylene glycol succinate on 80/100 mesh GAS-CHROM P (Applied Science Laboratories, Inc., Inglewood, CA). Post-column flow was split 7:1, and individual fatty acids plus all remaining areas of the chromatogram were trapped in 25 cm x 1.2 mm id glass capillary tubes packed in solid CO_2. Contents of the tubes were flushed with 1.0 ml toluene and counted in toluene fluor at an average counting efficiency of 87%. All trapping trials were done in duplicate, and relative radioactivity percentages of the trapped fractions agreed to within 10%.

RESULTS

Fatty Acid Oxidation and Incorporation

An average of 35% of administered [1-^{14}C]-linolenic acid was recovered as $^{14}CO_2$ in 24 hr. This percentage did not increase appreciably with experimental trials longer than 24 hr.

Liver and heart lipids incorporated much more radioactivity than gill lipids. However, while the amount of label in liver lipid decreased steadily after 24 hr, gill and heart lipids showed no clear pattern of label loss, and variability in those tissues was greater than in liver.

Composition and Turnover of Liver Phospholipids

Phospholipids comprised ca. 60% of the total liver lipids of coho salmon, the major components being CGP and EGP which collectively represented 80% of total phospholipid (Table I).

Comparison of the mean specific activities of the major liver phospholipids over time (Fig. 1) shows that, initially, CGP and EGP were the most highly labeled components. Calculation of halflives of these phospholipids from semilog plots indicated that sphingomyelin (S) and inositol glycerophospholipids (IGP) were turning over at the greatest rate, followed by CGP, serine glycerophospholipids (SGP), EGP and phosphatidic acid (PA) (Table I). All phospholipids steadily decreased in specific activity with time except diphosphatidylglycerol. The loss of label from the phospholipids appeared to take place at a much slower rate than the incorporation. Semilog plots of specific activity of total lipid, CGP and EGP of liver (Fig. 2) show linear relationships over time, indicating that disappearance of label from the acyl portions of the phospholipids is a logarithmic (halflife) function.

Acyl Composition of CGP, EGP, Diacylglycerol and Cholesteryl Esters

Acyl group analysis of CGP and EGP (Table II) revealed that CGP contained more 14:0 and 16:0 than did EGP, and that the reverse was true for 18:1 and 20:1. EGP also contained slightly greater amounts of 20:5n-3 and 22:6n-3, and in general was more unsaturated

than its choline counterpart, as shown by the unsaturation indices. Diacylglycerols contained more shorter chain fatty acids and much less 22:6n-3 than the phospholipids. Acyl composition of cholesteryl esters of liver tissue and blood plasma were similar. The major fatty acids in both tissues were 16:0, 18:1 and 22:6n-3. The content of the latter showed much variation, ranging from 15 to 50% of the fatty acids esterified to cholesterol. The higher values are in agreement with those reported for rainbow trout lipoprotein (22). In the present study, cholesteryl ester content of liver lipid averaged 0.20 mg per mg total lipid.

Turnover of Acyl Groups of CGP and EGP

The percentage of label found in the acyl portion of both EGP and CGP averaged 96% of that in the total phospholipid molecules, and this amount did not change appreciable with time. Considerable radioactivity was associated with 18:3n-3 at the earlier times, but the weight percent of this fatty acid was negligible and specific activities could not be calculated.

The change in specific activity over time of the three major n-3 fatty acids of hepatic CGP and EGP (20:5n-3, 22:5n-3, and 22:6n-3) is shown in Figure 3. Variations between individual fish account for the deviations, which generally diminish with time. The specific activity of 22:6n-3 was less than that of all other n-3 fatty acids at all times. All n-3 fatty acids decreased in specific activity between 120 and 240 hr, indicating that peak incorporation had passed and that halflives could be calculated from the rate of label depletion during this period. Comparison of the halflives of the n-3 fatty acids is shown in Table III. The halflife of each n-3 homolog was longer in EGP than in CGP, and the general pattern of fatty acid halflives relative to each other was the same in both phospholipids with the exception of 18:4n-3 (Table III). A sharp decrease in specific activity occurred between 20:4n-3 and 20:5n-3 in both phospholipids, and was still evident at 240 hr. The specific activities and halflives of 20:5n-3, 22:5n-3, and 22:6n-3 in diacylglycerol were not significantly different from those found in phospholipid. Surprisingly, no radioactivity was associated with any fatty acids esterified to cholesterol in liver lipid, despite the high content of 22:6n-3.

DISCUSSION

Fatty Acid Oxidation and Incorporation

The recovery of significant amounts of radioactivity in expired carbon dioxide is expected since carp mitochondria have been shown to oxidize linolenic acid at a faster rate than 20:5n-3, 22:6n-3, 18:1n-9, and 16:0 (23). The fact that most of the labeled CO_2 was recovered during the first 24 hr indicates that it was the initially available linolenate that was being oxidized, not labeled acids hydrolyzed from phospholipids or acylglycerols. Similar observations were made by Owen et al. (14), who noted that $^{14}CO_2$ expired by turbot following labeled 18:3n-3, 18:2n-6, or 18:1n-9 intake occurred primarily during the first 24 hr.

Turnover of Phospholipids

The relative turnover pattern of four major fish liver phospholipids (IGP>CGP>SGP>EGP) is similar to that observed by Smith and Eng (24) in rat myelin and by Anderson (25) in goldfish liver mitochondria following ^{14}C-acetate labeling. De Tomas and Mercuri (26) also found that label from [1-^{14}C]linolenic

TABLE I

Composition and Halflives of Phospholipids of Coho Salmon Liver

Phospholipid	Percent[a] ± std. dev.	Halflife[b] (hr)
Choline glycerophospholipids	56.9 ± 3.5	69
Ethanolamine glycerophospholipids	20.4 ± 2.1	107
Inositol glycerophospholipids	6.3 ± 0.8	51
Sphingomyelin	4.7 ± 1.0	54
Serine glycerophospholipids	3.3 ± 0.6	94
Diphosphatidylglycerol	2.1 ± 0.4	n.d.[c]
Phosphatidic acid	0.6 ± 0.3	134
Lysophosphatidylcholine	1.3 ± 0.6	n.d.
Lysophosphatidylethanolamine	1.2 ± 0.6	n.d.
Σ	96.8	

[a]Percent of total lipid phosphorous, mean of nine fish.

[b]Calculated on basis on lipid phosphorus.

[c]Not determined.

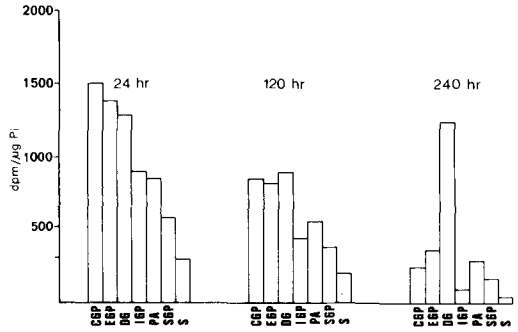

FIG. 1. Distribution of radioactivity in liver phospholipids following injection of [1-^{14}C] linolenic acid. Each bar represents the mean of three experiments. CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; DG, diphosphatidyl glycerol; IGP, inositol glycerophospholipids; PA, phosphatidic acid; SGP, serine glycerophospholipids; S, sphingomyelin.

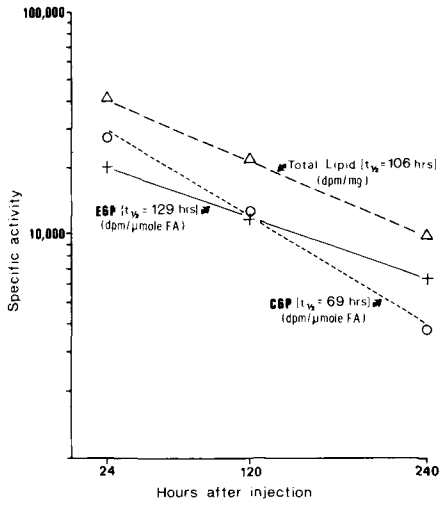

FIG. 2. Loss of radioactivity from liver lipids following injection of [1-^{14}C] linolenic acid. Specific activity units are: total lipid, dpm/mg lipid; CGP and EGP, dpm/μmole acyl groups. Each point represents mean of three fish.

TABLE II

Acyl Composition of Choline and Ethanolamine Glycerophospholipids and Diacylglycerols from Liver of Coho Salmon[a]

Fatty acid	Percent ± std. dev. Choline glycerophospholipids	Ethanolamine glycerophospholipids	Diacylglycerol
14:0	1.4 ± 0.6	0.3 ± 0.1	2.3 ± 0.5
14:1	0.2 ± 0.0	0.3 ± 0.1	0.7 ± 0.2
16:0	20.5 ± 3.8	9.7 ± 1.1	17.5 ± 3.9
16:1	3.7 ± 0.9	1.2 ± 0.3	4.5 ± 1.5
18:0	7.3 ± 1.1	6.5 ± 0.9	8.7 ± 1.8
18:1	13.9 ± 2.6	20.3 ± 2.3	22.9 ± 7.8
18:2n-6	0.9 ± 0.2	1.4 ± 0.4	2.3 ± 0.8
20:1	0.8 ± 0.3	2.2 ± 0.4	1.7 ± 0.5
18:4n-3	0.6 ± 0.2	0.8 ± 0.3	1.2 ± 0.8
20:2n-9	1.6 ± 1.0	0.8 ± 0.7	---
20:3n-6 + 22:1n-9	0.2 ± 0.1	0.3 ± 0.1	trace
20:4n-6	3.8 ± 0.8	1.1 ± 0.2	3.4 ± 1.6
20:4n-3	0.4 ± 0.3	0.6 ± 0.3	2.9 ± 0.6
20:5n-3	5.7 ± 0.8	8.2 ± 1.7	7.9 ± 3.1
22:5n-3	2.3 ± 0.5	2.7 ± 0.4	3.3 ± 1.4
22:6n-3	36.4 ± 3.6	42.8 ± 2.8	18.7 ± 6.6
Σ	99.7	99.2	98.0
U.I.[b]	302	351	233
Total n−6	4.9	2.8	5.7
Total n-3	45.4	55.1	34.0
n-3/n-6	9.3	19.7	6.0

[a]Choline and ethanolamine glycerophospholipids, average of nine fish; diacylglycerol, average of four fish. The following fatty acids were present only in trace amounts: 18:3n-3, 22:4n-6, 22:4n-3 and 22:5n-6.

[b]U.I. = unsaturation index, defined as Σ(number of double bonds in each fatty acid) x (% of each fatty acid).

acid accumulated to a greater extent in CGP than in EGP in rat liver lipid. The halflives of total lipid, CGP, and EGP (106, 69, and 107 hr, respectively) approximate those found by Omura et al. (27) for total lipid and total phospholipid of rat endoplasmic reticulum (97 and 56-79 hr, respectively) following ^{14}C acetate labeling. In contrast, calculations from the data of Poovaiah et al. (6) show a halflife of ca. 9 hr for rat liver CGP and EGP after ^{14}C linolenic acid injection.

There are two lines of evidence from the present study which together indicate that most of the n-3 fatty acids in liver phospholipid were incorporated by selective acyl transfer. These are (1) the low amount of diacylglycerol relative to phospholipid, and (2) the similarity of specific activity and halflife of the n-3 fatty acids between phospholipid and diacylglycerol. It is apparent that only a small portion of the labeled n-3 fatty acids found in CGP and EGP were delivered via de novo synthesis of those phospholipids.

Fatty Acid Composition, Incorporation, and Turnover

Coho salmon liver EGP contains less palmitate and arachidonate, and more oleate than CGP. The opposite is true of rat liver (28). However, EGP of both rat and salmon contains more 22:6n-3 and is generally more unsaturated than CGP.

Data in Table III show that the incorporation and turnover pattern of the n-3 fatty acids in choline and ethanolamine glycerophospholipids is complex. The specific activity of 20:5n-3 of EGP is equal to that of CGP at 120 hr, even though its precursors (18:4n-3 and 20:4n-3) in EGP have only half the activity of their counterparts in CGP. This may indicate some transfer of 20:5n-3 from CGP to EGP, through acyl or base exchange. A second possible explanation follows from Holub and Kuksis (29), who found that 16:0-containing phospholipid species incorporated linoleic acid to a much greater extent than other molecular species, possibly reflecting both reacylation and de novo phospholipid synthesis. In the present study, CGP is the major 16:0-containing lipid, and indeed shows much higher specific activities in the shorter n-3 fatty acids. Several studies (7,29,30) have shown that the longer polyunsaturated fatty acids (20:4n-6, 20:5n-3, 22:6n-3) are incorporated into phospholipids primarily by reacylation rather than via de novo synthesis. The much higher specific activities of the shorter, less unsaturated n-3 homologs is in accordance with suggestions by these authors that different (or multiple) pathways may be operable for incorporation of these fatty acids as opposed to the desaturation-elongation products.

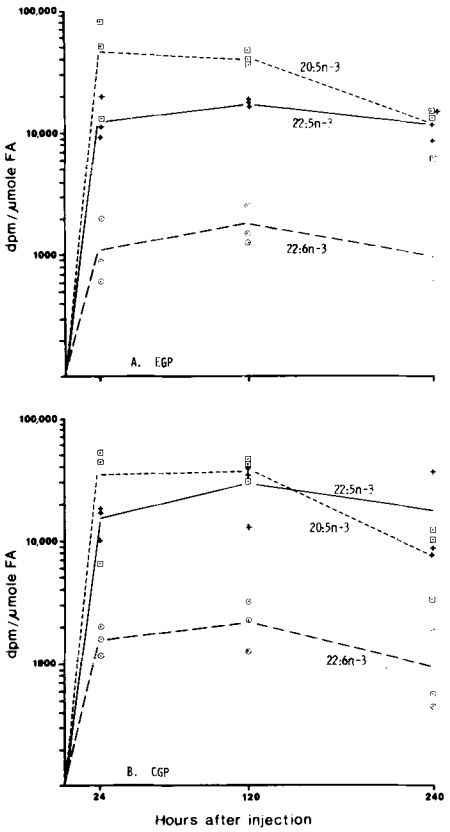

FIG. 3. Specific activity of fatty acids of ethanolamine and choline glycerophospholipids of liver at various times after injection with [1-^{14}C] linolenic acid. Each point represents one fish.

The general magnitude of the acyl halflives reported here resemble those that may be calculated from the data of van Golde et al. (31), who examined the rates of replacement of acyl groups in the 2-position of liver CGP in essential fatty acid deficient rats following EFA feeding. The approximate halflives of 20:3n-9 and its replacement 20:4n-6 were 48 and 95 hr, respectively, during the initial 120 hr.

Continuous feeding trials with coho salmon (32), in vitro competitive inhibition studies (33), and the present data on halflives indicate that the desaturation of 18:3n-3 and 22:5n-3 may be key points in the control of long chain n-3 content of phospholipids. The apparent high metabolic activity of 20:5n-3 merits further investigation as does the high 22:6n-3 content of cholesteryl esters in regards to the role of the latter in essential fatty acid trans-

TABLE III

Comparison of Halflives and Specific Activity of Fatty Acids in Choline and Ethanolamine Glycerophospholipids of Liver

	Choline glycerophospholipids			Ethanolamine glycerophospholipids		
		dpm/μmole (x10^3)[a]			dpm/μmole (x10^3)[a]	
Fatty acid	t½(hr)	120 hr	240 hr	t½(hr)	120 hr	240 hr
18:4n-3	64	237	65.5	172	138	85.0
20:4n-3	80	306	109	96	139	57.6
20:5n-3	53[b]	40.0	7.2	65[d]	40.5	11.5
22:5n-3	168[bc]	29.0	17.0	192[e]	17.6	11.5
22:6n-3	100[c]	2.2	0.9	128[f]	1.8	0.9
20:4n-6	70	14.6	4.4	307	43.8	34.0

[a]Means of three fish. Halflives with different superscripts in the same column are different by t-test ($P \leq 0.02$).

port and storage. The observation that no label was found in n-3 fatty acids of cholesteryl esters indicates the presence of a stable pool of essential fatty acids in the liver. Evidence that the polyunsaturated acyl groups of CGP are turning over at rates different from those of EGP may reflect: (a) acyltransferase specificity for not only fatty acids but also phospholipids, or (b) different activities of phospholipid exchange proteins. Such specificity would undoubtedly play a basic role in influencing phospholipid and acyl composition of fish membranes.

ACKNOWLEDGMENTS

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Interaction of Human Plasma High Density Lipoprotein HDL_2 with Synthetic Saturated Phosphatidylcholines[1]

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ABSTRACT

The interaction of human plasma high density lipoprotein HDL_2 (d 1.063-1.125 g/ml) with sonicated dispersions of synthetic saturated phosphatidylcholines, dipalmitoyl- ($diC_{16}PC$), dimyristoyl- ($diC_{14}PC$), didodecanoyl- ($diC_{12}PC$), didecanoyl- ($diC_{10}PC$), and dioctanoyl- (diC_8PC) L-alpha phosphatidylcholine, was investigated. Incubation (4.5 hr, 37 C) of HDL_2 with $diC_{14}PC$, $diC_{12}PC$, $diC_{10}PC$ and diC_8PC followed by gradient gel electrophoresis or preparative ultracentrifugation resulted in a redistribution of apolipoprotein A-I (apoA-I). The extent of redistribution depended on the molar ratio of the phospholipid to HDL_2 in the incubation mixture. Redistributed apoA-I occurred as lipid-free apoA-I and/or as complexes of apoA-I with phosphatidylcholine. Increasing the length of time of ultracentrifugation of the interaction mixtures did not increase the extent of redistribution. No redistribution of apoA-I was detected following incubation and gradient gel electrophoresis or preparative ultracentrifugation of mixtures of HDL_2 with dispersions of $diC_{16}PC$.

INTRODUCTION

The synthetic saturated phospholipid, dimyristoylphosphatidylcholine ($diC_{14}PC$), has been extensively used as a model compound in physical-chemical studies of the interaction of phospholipids with human plasma lipoproteins (1-3) and their apolipoproteins (4-6). Interaction between multilamellar liposome preparations of $diC_{14}PC$ and high density lipoproteins (HDL) has been shown to result in the formation of (a) discoidal complexes of $diC_{14}PC$ and apolipoprotein A-I (apoA-I) and (b) residual HDL of increased size and decreased density (1). In studies performed at low levels of $diC_{14}PC$ relative to those of HDL_{2b} (d 1.063-1.100 g/ml, a subclass of HDL), we reported uptake of $diC_{14}PC$ by the HDL_{2b} and redistribution of some apoA-I from the HDL surface upon preparative ultracentrifugation or gradient gel electrophoresis of the incubation mixtures (3). The amount of apoA-I redistributed corresponded to about 1-2 apoA-I molecules per HDL_{2b} molecule. The present study compared apolipoprotein redistribution following preparative ultracentrifugation and gradient gel electrophoresis of incubated mixtures of HDL_2 (d 1.063-1.125 g/ml) with synthetic saturated phosphatidylcholines which differed in acyl chain length.

EXPERIMENTAL PROCEDURES

Preparation of HDL_2

Blood was drawn from normal fasting subjects (40-45 yr) into evacuated tubes containing EDTA as anticoagulant. The plasma was isolated by centrifugation and ethylmercurisalicylic acid (0.124 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml) were added. The same concentration of these reagents was used in all subsequent procedures. To remove the d $\leqslant$ 1.063 g/ml fraction, solid sodium bromide was added to the plasma and 4 ml were layered under a NaBr solution to raise the background density. Following a 24 hr ultracentrifugation (50.3 Ti rotor, Beckman Instruments, Inc., Palo Alto, CA; 179,000 x g, 17 C), the d $\leqslant$ 1.063 top 1 ml fraction was removed and the subnatant adjusted with a NaBr solution for ultracentrifugal isolation of the HDL_2 fraction. The ultracentrifugal conditions were the same as stated above except for the time period which, in this case, was 40 hr. The top 2 ml contained the HDL_2, d 1.063-1.125 g/ml fraction, and was dialyzed to 5 mM NH_4HCO_3, pH 8.6, prior to incubation procedures.

Preparation of Phospholipid Dispersions

$DiC_{16}PC$ and $diC_{14}PC$ were obtained from Sigma Chemical (St. Louis, MO) and were of 98% purity. The $diC_{12}PC$, $diC_{10}PC$ and diC_8PC from Supelco, Inc. (Bellefonte, PA) were 99% pure. The dispersions were prepared in 5 mM NH_4HCO_3 by sonication using a Branson Sonifier Cell Disrupter (Model W185) with a microprobe. Each phospholipid was sonicated above its transition temperature for 10-15 min at a maximum energy level of 50 W. When analyzed by thin layer chromatography, the sonicated phospholipids each gave a single spot corresponding to the position of the unsonicated phospholipid. Negative stain

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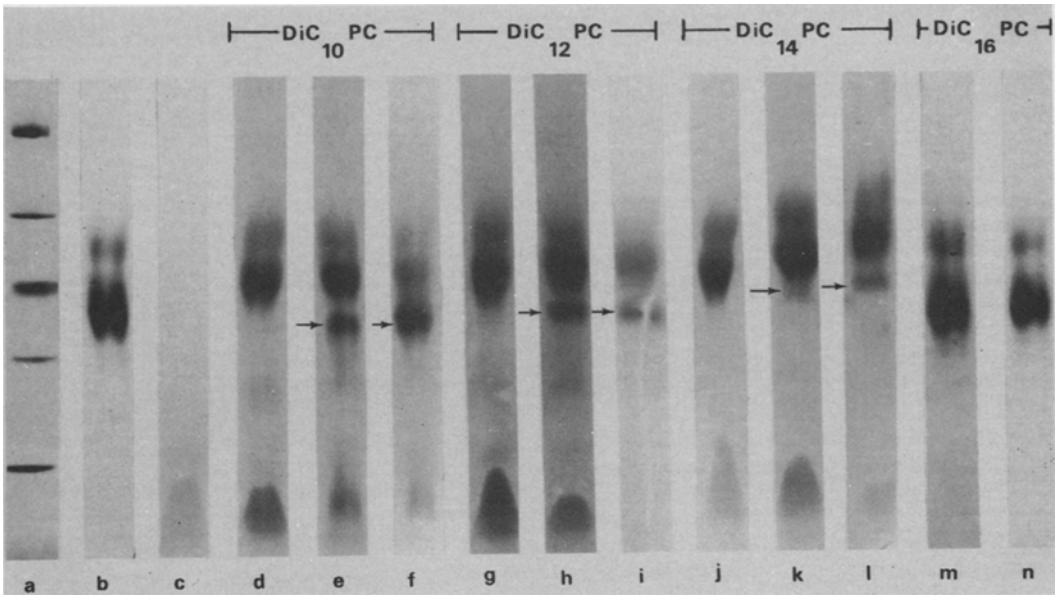

FIG. 1. Gradient gel electrophoretograms of: (a) reference proteins (top to bottom; diameter) thyroglobulin (13.2 nm), apoferritin (11.8 nm), catalase (10.4 nm), lactate dehydrogenase (9.0 nm), bovine serum albumin (7.2 nm); (b) control HDL_2; (c) apoA-I; (d) 147:1, (e) 221:1, (f) 294:1, $diC_{10}PC/HDL_2$ molar ratio; (g) 132:1, (h) 206:1, (i) 279:1, $diC_{12}PC/HDL_2$ molar ratio; (j) 161:1, (k) 235:1, (l) 309:1, $diC_{14}PC/HDL_2$ molar ratio; (m) 265:1, (n) 397:1, $diC_{16}PC/HDL_2$ molar ratio. The arrows indicate new bands appearing after incubation of HDL_2 with the phosphatidylcholines.

electron microscopy (3) showed the diC_8PC sonicated dispersion to consist primarily of amorphous micellar structures. The $diC_{14}PC$ and $diC_{16}PC$ dispersions contained a majority of single-bilayer liposomes. The predominant physical structures identified in the $diC_{10}PC$ and $diC_{12}PC$ dispersions were multilamellar liposomes. A multilamellar liposomal dispersion of $diC_{14}PC$ was also prepared using the above sonication conditions but for a shorter period of time (5 min).

Incubation Procedures

All samples were incubated at 37 C in a shaking water bath for 4.5 hr under nitrogen in 5 mM NH_4HCO_3.

Gradient Gel Electrophoresis and Preparative Ultracentrifugation

Gradient gel electrophoresis was performed on a Pharmacia Electrophoresis Apparatus GE-4 using PAA 4/30 gradient gels, according to Anderson et al. (7). The gels were calibrated with reference proteins from the Pharmacia high molecular weight Electrophoresis Calibration Kit (Pharmacia Fine Chemicals, Uppsala, Sweden). Particle sizes were estimated from a curve of migration distance of the reference proteins vs. their Stokes' diameter.

Following interaction of HDL_2 with the phospholipid dispersions, the $d \leqslant 1.20$ g/ml and $d > 1.20$ g/ml fractions were separated by preparative ultracentrifugation (50.3 Ti rotor, Beckman Instruments; 179,000 x g, 48 hr).

Chemical and Other Analyses

Protein and phosphorus analyses on all control preparations, incubated mixtures and ultracentrifugal fractions were performed according to Lowry et al. (8) and Bartlett (9), respectively. Polyacrylamide gel electrophoresis for apolipoprotein identification followed the method of Kane (10).

RESULTS AND DISCUSSION

Gradient Gel Electrophoresis of Interaction Mixtures

Gradient gel electrophoresis (GGE) of control HDL_2 showed the presence of two banding maxima corresponding to two major HDL subclasses, HDL_{2b} and HDL_{2a} (7). Incubation (4.5 hr, 37 C) of control HDL_2 produced negligible change in its electrophoretogram. GGE patterns (Fig. 1) of incubated mixtures of HDL_2 plus increasing concentrations of $diC_{10}PC$, $diC_{12}PC$ and $diC_{14}PC$ dispersions

showed the appearance of a new band of material corresponding to particles with diameters somewhat smaller (6.1 nm) than bovine serum albumin (7.2 nm). Polyacrylamide gel electrophoresis of this material after isolation by preparative ultracentrifugation showed it to exhibit migration properties of apoA-I (10). No material was observed in this size range in incubation mixtures containing dispersions of $diC_{16}PC$. At the highest molar ratios of phospholipid to HDL_2 investigated, the staining intensity of this band was less than observed at the lower ratios. Our earlier studies on the interaction of HDL_{2b} with liposomes of $diC_{14}PC$ established that such decrease in staining intensity reflected a complexing of the apoA-I with the phospholipid to form, in the case of $diC_{14}PC$, discoidal particles of dimensions 10.0 x 4.4 nm. Such complexes were noted in the present experiments using HDL_2, as evidenced by the new band (at approximately the migration distance of catalase; see Fig. 1, arrows) which appeared in mixtures at the higher molar ratios ($\geq$ 235:1) of $diC_{14}PC$ to HDL_2. Similarly, new bands, corresponding to particles somewhat smaller (9.6 nm) than catalase (10.4 nm), appeared in the electrophoretograms of interaction mixtures at higher molar ratios ($>$ 200:1) of either $diC_{10}PC$ or $diC_{12}PC$ to HDL_2. Electrophoretic patterns of incubation mixtures containing diC_8PC (Fig. 2) also showed new bands which corresponded to apoA-I and apparently to apoA-I-phospholipid complexes of substantially smaller size (7.4 nm) than observed with the other phospholipids. In general, the above data indicated comparable interaction products for all the phospholipids except $diC_{16}PC$. These products included apoA-I at lower molar ratios of PC to HDL_2 and complexes of apoA-I with PC when the molar ratio was further increased in the incubation mixture. The electrophoretograms of Figures 1 and 2 also indicated an increase in HDL_2 particle size as determined by the change in migration distance of the major HDL_2 band. Separate isolation and identification of complexes of apoA-I with $diC_{12}PC$, $diC_{10}PC$ and diC_8PC, formed at the higher molar ratios, was not performed. However, model complexes resulting from interaction of purified apoA-I with dispersions of these phospholipids were analyzed by GGE and were found to migrate to positions identical to those of bands noted above as representing apoA-I-phospholipid complexes.

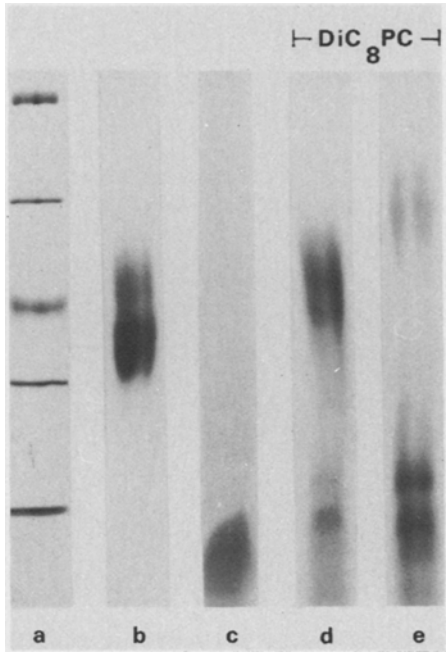

FIG. 2. Gradient gel electrophoretograms of: (a) reference proteins (see Fig. 1); (b) control HDL_2; (c) apoA-I; (d) 235:1, (e) 324:1, diC_8PC/HDL_2 molar ratio.

Ultracentrifugal Analysis of Interaction Mixtures

Preparative ultracentrifugation of the incubation mixtures at d 1.21 g/ml separated lipid-bound apolipoprotein of d $\leq$ 1.20 g/ml from lipid-free apolipoprotein and/or phospholipid-apolipoprotein complexes of d $>$ 1.20 g/ml. Figure 3 shows the change in percent of total HDL_2 protein in the d $>$ 1.20 g/ml fraction with increasing molar ratio of phospholipid to HDL_2. The amount of protein in the d $>$ 1.20 g/ml fraction increased to a maximum value and then decreased as the molar ratio was further increased. The amount of d $>$ 1.20 g/ml protein at each ratio (for a particular concentration of HDL_2) did not increase with further increase in the time (from 48 to 60 hr) of ultracentrifugation, indicating that further redistribution of apoA-I by mass action during ultracentrifugation, did not occur. No difference in the percent protein redistributed into the d $>$ 1.20 g/ml fraction was observed for the same molar ratios when a multilamellar liposomal dispersion of $diC_{14}PC$ was used in place of the predominantly single-bilayer liposomal dispersion of $diC_{14}PC$.

Some phospholipid was detected in the d $>$ 1.20 g/ml fractions from mixtures containing $diC_{12}PC$, $diC_{10}PC$ and diC_8PC; none was detected with $diC_{14}PC$ or $diC_{16}PC$. The phospholipid values in these d $>$ 1.20 g/ml fractions followed the rise and fall of the protein

values in the same fractions. At maximum values of protein in the d > 1.20 g/ml fraction, the average weight ratio of phospholipid to protein was 0.04:1 ($diC_{12}PC$), 0.21:1 ($diC_{10}PC$), and 0.86:1 (diC_8PC). These data indicated that some partial complexing of apoA-I had occurred. For each of the phospholipids, the percent of HDL_2 protein in the d > 1.20 g/ml fraction decreased markedly at higher molar ratios, suggesting that sufficient phospholipid had complexed with the protein to cause its flotation into the d ⩽ 1.20 g/ml fraction. By electrophoresis, the latter complexes showed particle sizes in the range of 9.6-10.4 nm (for $diC_{14}PC$, $diC_{12}PC$ and $diC_{10}PC$) and 7.1-7.8 nm (for diC_8PC). The d > 1.20 g/ml complexes detected by preparative ultracentrifugation of mixtures of HDL_2 and dispersions of $diC_{12}PC$ and $diC_{10}PC$, at molar ratios associated with maximum protein redistribution, were not visualized as separate new bands by GGE. The concentration of these new complexes was either too low or they were part of the material considered to be apoA-I (in multimeric form). Since our estimate of maximal redistributed protein was derived from the amount of protein in the d > 1.20 g/ml fraction, some underestimation was possible because part of the protein might have been more fully complexed by the phospholipid, and thereby shifted into the d ⩽ 1.20 g/ml fraction. We used analytic ultracentrifugation (11) to check for the presence of complexes in the d ⩽ 1.20 g/ml fraction of interaction mixtures containing $diC_{12}PC$ and $diC_{10}PC$. At molar ratios associated with maximum redistribution, no new peaks were observed in the schlieren patterns which correlated with apoA-I-phospholipid complexes; only a shift to faster flotation rates and an increase in area of the major HDL_2 peak were seen. These latter changes were consistent with uptake of phospholipid by HDL_2 and possibly some loss of apoA-I (3). At even higher molar ratios, new peaks appeared in the flotation rate regions expected for d ⩽ 1.20 g/ml complexes composed of apoA-I and the corresponding phospholipid. Thus, we consider our estimates of maximal redistributed protein not grossly in error due to incomplete recovery of protein not bound to HDL_2 lipid. The maximum amount of protein redistributed into the d > 1.20 g/ml fraction under the experimental conditions used was found to be: 17% ($diC_{14}PC$), 34% ($diC_{12}PC$ and $diC_{10}PC$) and 48% (diC_8PC) of the total HDL_2 protein.

Comparable studies were also performed on incubation mixtures containing HDL_2 and dispersions of $diC_{16}PC$. However, since the incubations with the other phospholipids were performed at a temperature (37 C) above their transition temperatures, we evaluated the interaction of HDL_2 with $diC_{16}PC$ at 42 C, slightly above the latter's transition temperature. We found no evidence of apoA-I redistribution upon preparative ultracentrifugation of the mixtures, no change in analytic ultracentrifugal pattern, nor any increase in HDL_2 particle size upon GGE.

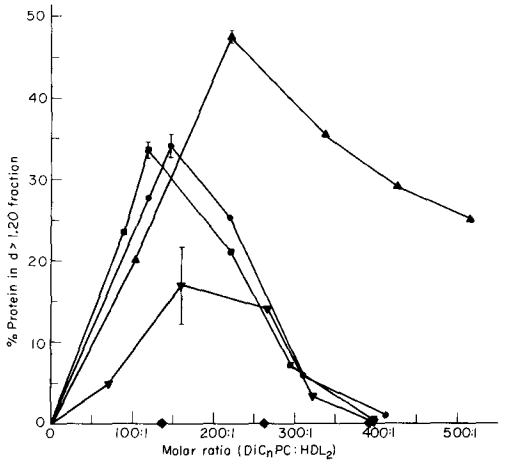

FIG. 3. Protein recovered in the d > 1.20 g/ml fraction following preparative ultracentrifugation of interaction mixtures of phospholipids and HDL_2 as a function of initial mixture composition (molar ratio, phospholipid/HDL_2): diC_8PC + HDL_2 (▲ – ▲), $diC_{10}PC$ + HDL_2 (● – ●), $diC_{12}PC$ + HDL_2 (■ – ■), $diC_{14}PC$ + HDL_2 (▼ – ▼), and $diC_{16}PC$ + HDL_2 (◆ – ◆). Standard deviations at maximal protein values are shown. Conditions of incubation and ultracentrifugation are described in Experimental Procedures.

In summary, substantial differences in the extent of redistribution of apoA-I were observed following incubation and ultracentrifugal fractionation of mixtures of HDL_2 and dispersions of different acyl chain length phosphatidylcholines. For phosphatidylcholine dispersions consisting primarily of multilamellar liposomes ($diC_{10}PC$ and $diC_{12}PC$), the extent of redistribution was approximately two-fold that observed with either multilamellar or single-bilayer liposomal dispersions of $diC_{14}PC$. Interaction of HDL_2 with single-bilayer liposomal dispersions of $diC_{16}PC$ resulted in no detectable redistribution of apoA-I. The micelle-forming phosphatidylcholine (diC_8PC) was associated with a three-fold greater redistribution of apoA-I when compared to the $diC_{14}PC$ mixture, under the experimental conditions used.

Clearly, the interactions which lead to redistribution of apoA-I from the HDL_2 surface to

lipid-free apoA-I or apoA-I-phospholipid complexes, are complex, and specific mechanisms controlling apoA-I dissociation are yet to be fully defined. Nevertheless, the present work indicates a high reactivity of HDL_2 with synthetic saturated phosphatidylcholines ($diC_{14}PC$, $diC_{12}PC$, $diC_{10}PC$ and diC_8PC) which appears related to acyl chain length and results in extensive redistribution of its major structural apolipoprotein, apoA-I.

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Separation of Oxidized and Unoxidized Molecular Species of Phosphatidylcholine by High Pressure Liquid Chromatography[1]

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ABSTRACT

Soy phosphatidylcholine (PC) has been separated into its major molecular species by reverse-phase high pressure liquid chromatography (HPLC). An aqueous methanol gradient was used that allowed detection of the various species by their absorbance at 206 nm. Oxidized species were detected by their absorbance at 234 nm and were resolved from the unoxidized species. This technique has been used to separate and purify unoxidized dilinoleyl phosphatidylcholine (di 18:2 PC) from other species of soy PC and to monitor the autoxidation of an aqueous suspension of the purified di 18:2 PC. Two oxidized products were formed from di 18:2 PC. Further analysis showed that they were PC, but one of the products contained an oxidized and an unoxidized fatty acid; in the other product, both fatty acids were oxidized.

INTRODUCTION

Although high pressure liquid chromatography (HPLC) has found widespread use for separating many organic compounds, there is only a limited number of reports on the HPLC separation of phospholipids. A major deterrent to the use of HPLC as an analytical technique for lipid analysis has been the problem of rapidly detecting small amounts of phospholipid in the eluant. The 200 nm range of phospholipid absorbance limits the choice of solvents to those which do not absorb in that region, although several solvent systems have been found which allow ultraviolet (UV) detection and provide resolution of phospholipid classes by HPLC on silicic acid (1,2) or μBondapak-NH_2 columns (3).

We have been studying oxidized PC from soy (4,5) and have found that it contributes to the bitter flavor of soy meal. In order to minimize autoxidation during isolation and to facilitate studies on the oxidiation of molecular species, we wanted a rapid method that could separate oxidized from unoxidized phosphatidylcholine (PC). The resolution of compounds by reverse-phase chromatography is due, in part, to the nonionic interactions of molecules with the stationary support; therefore, this chromatographic technique could provide a method to separate oxidized from unoxidized species of PC. Porter et al. (6) have recently reported the reverse-phase separation of several PC molecular species, but the inclusion of chloroform in the solvent only allows detection by refractive index, which precludes gradient elution and 206 nm detection.

Arvidson (7) has reported the reverse-phase separation of egg PC molecular species on an alkylated derivative of Sephadex in a methanol-water solvent system. We report here the separation of soy PC molecular species and the separation of oxidized from unoxidized species by reverse-phase HPLC in aqueous methanol while detecting the lipid species by their UV (206 nm) absorbance.

MATERIALS AND METHODS

Soy PC was purified from commercial lecithin (Central Soya, Chicago, IL) by chromatography on Florisil and DEAE-cellulose columns (Supelco Inc., Bellefonte, PA) as described by Sessa et al. (4). Thin layer chromatography (TLC) was carried out on Silica Gel 60 plates (EM Laboratory, Inc., Elmsford, NY) with $CHCl_3/CH_3OH/H_2O$ (65:35:4), and the lipids were visualized with 380 nm light after spraying with 0.1% 8-anilino-1-napthalene sulfonate in H_2O (8).

Reverse-phase chromatography was carried out on a μBondapak C_{18} column (3.9 mm x 30 cm) in aqueous methanol, either isocratically (95%) or with a linear gradient (91 to 95%) at 2 ml/min. Equipment for HPLC (Waters Assoc., Inc., Milford, MA) included model 6000-A pumps, UK6 injector, column, model 450 detector, and model 660 solvent programmer. Lipids were detected by their absorbance at 206 or 234 nm.

Fractions were collected from the HPLC eluant, chloroform was added to make them 2:1 in $CHCl_3/CH_3OH$, then sufficient 1.0% NaCl was added to give 0.2 vol of aqueous phase (9). The organic phase was separated, washed with water, then stored at -10 C until analyzed.

Samples of the isolated fractions were

[1]Present in part at FASEB 63rd Annual Meeting, Dallas, Texas, April 1979.

transmethylated with 0.5 M KOH in anhydrous methanol, neutralized with glacial acetic acid, and after addition of H_2O, the methyl esters were extracted with petroleum ether/diethyl ether (1:1). Part of each sample that contained oxidized fatty acids was transmethylated in the presence of $NaBH_4$. The methyl esters were analyzed on a Packard 428 gas chromatograph using a 6 ft x 2 mm glass column packed with 10% DEGS or a 3 ft x 2 mm glass column packed with 5% Apiezon L (Supelco Inc., Bellefonte, PA).

Oxidized fatty acid methyl esters were analyzed by gas chromatography-mass spectroscopy (GC-MS) after silylation as described elsewhere (10).

Dilinoleyl phosphatidylcholine (di 18:2 PC) was prepared in the following manner. The eluant from HPLC containing di 18:2 PC was collected and combined from a series of runs of soy PC and then extracted as described above. This material was rechromatographed, reextracted into chloroform, and used for the oxidation studies.

The di 18:2 PC was oxidized as an aqueous suspension. The lipid was taken to dryness under nitrogen; then water was added to make the suspension 1 μmole/ml. This suspension was mixed vigorously for 5 min and then stirred gently in air at room temperature. Aliquots (25 μl) were analyzed by HPLC at various time intervals to monitor the reaction; after oxidizing for 60 hr, the lipids were extracted with $CHCl_3/CH_3OH$ (2:1). The extract was then chromatographed by reverse-phase HPLC in 90% CH_3OH, and the separated oxidized products and unoxidized material were collected. Collected fractions were taken to dryness in vacuo with addition of CH_3OH and then stored at -10 C in $CHCl_3$ until analyzed.

RESULTS AND DISCUSSION

When soy PC is chromatographed by reverse-phase in a gradient of methanol/water, it is resolved into a number of species as shown in Figure 1.

Fatty acid analysis of the material in the major peaks indicated in Figure 1-A is given in Table I, along with the major molecular species found in each peak. Peak V contains a number of fatty acids other than 18:3, which may be derived from contaminating oxidized species (see below). Peak VI appears to be pure 18:2-18:3 PC, but if the sample is contaminated by equal amounts of di 18:3 from the preceding peak and di 18:2 from the following peak, the contamination would be impossible to detect by fatty acid analysis. The di 18:2

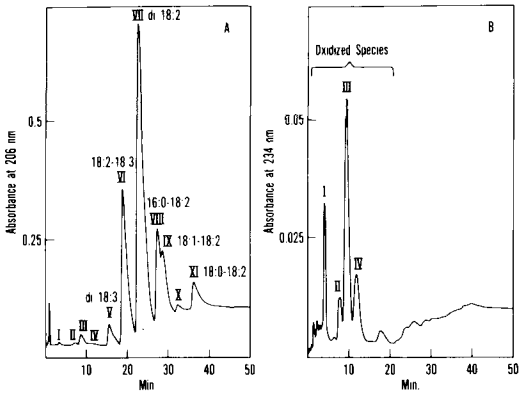

FIG. 1. Elution profile of UV absorbing species of soy PC. Conditions: 2.5 mg PC, μBondapack C_{18} eluted at 2 ml/min with 91% aqueous CH_3OH for 15 min, then with a linear gradient to 95% methanol for 20 min. A, absorbance at 206 nm, B, absorbance at 234 nm.

fraction (Peak VII) appears to contain a small amount of 16:0-18:3 species even after rechromatography on reverse-phase HPLC.

No attempt was made to resolve the 16:0-18:2 species (Peak VIII) from the 18:1-18:2 species (Peak IX), but they may be separated on the Fatty Acid Analysis Column as described by Porter et al. (6) who report the separation of the analogous molecular species 16:0-18:1 from di 18:1. The component of Peak X was a mixture of all the fatty acids listed in Table I; due to the small amount found in soy PC, it was not studied further. Analysis of the fatty acids in the leading edge of Peak XI showed it to contain mostly 18:1, indicating that under these conditions di 18:1 PC may not be completely resolved from 18:0-18:2 PC.

Reverse-phase HPLC of fatty acid methyl esters (11) and triglycerides (12) has shown that species differing by two carbons can be separated and that a double bond decreases the retention time equivalent to a saturated species with two less carbons. This separation is also seen in our reverse-phase chromatography of phosphatidylcholine and, because of the comparatively simple fatty acid composition of soy PC, the major molecular species of soy PC can be resolved.

Figure 1-B shows the chromatogram of the same sample measuring the absorbance at 234 nm. Absorbance at 234 nm was taken as an indication of oxidized fatty acid moieties and by this criterion, the oxidized species of soy PC have been resolved from the unoxidized except in the area of the di 18:3. Analysis by GC-MS of the fatty acid methyl esters in the first

TABLE I

Mole Percent Fatty Acid Composition of the Soy PC Species Separated by Reverse-Phase HPLC

Peak[a]	16:0	18:0	18:1	18:2	18:3	Major species
V	5.0	1.5	2.7	3.2	87.0	di-18:3
VI				49.5	49.6	18:2-18:3
VII	2.2	0.9		93.2	3.6	di-18:2
VIII	39.2		8.9	51.7		16:0-18:2
IX	22.5		26.0	51.4		18:1-18:2
XI	0.8	43.8	7.3	45.4	2.6	18:0-18:2

[a]See Figure 1 for peaks analyzed.

four peaks showed that in addition to unoxidized fatty acids, there was a complex mixture of oxo, epoxy, and hydroxy fatty acids as previously described (5).

The di 18:2 PC fraction of soy PC was collected, then purified by rechromatography and allowed to undergo autoxidation in water. Figure 2-A and B show respectively the HPLC spectra before and after oxidation, indicating a decrease in unoxidized di 18:2 PC with the generation of two new species that have absorbance at 234 nm (Figure 2-C). TLC analysis of the compounds comprising the three peaks is shown in Figure 3, and although they cannot be separated by TLC under the conditions used, there is a slight difference in polarity in the order expected from the reverse-phase HPLC separation.

The total ionization chromatogram of the silylated methyl esters from the PC in Peak 2 (Figure 2) is presented in Figure 4. Mass spectra 93, 104, and 108 (indicated by arrows) are all similar with major ion peaks at 382, 311, 225, 130, and 73, indicating that the oxidized fatty acids are 9 or 13 hydroxy dienes (10,13). Their chromatographic behavior allows tentative identification as 9 or 13 *cis-trans* and 9 or 13 *trans-trans* hydroxy diene (13). GC-MS of the silylated methyl esters from the PC in Peak 1 (Figure 2) showed the same fragmentation pattern, except that the amount of unoxidized 18:2 was markedly reduced. By quantitation of the methyl esters on Apeizon L., the 18:2 to hydroxy diene ratio was found to be 1:1 in Peak 2 and 15:85 in Peak 1, which indicates that a single fatty acid is oxidized in the PC in Peak 2, but both fatty acids are oxidized in the PC in Peak 1.

The oxidized PC may be hydroperoxide as isolated, but lack of material has prevented a chemical determination. There was no qualitative difference between the oxygenated fatty acids extracted from untreated or $NABH_4$ treated samples; but more hydroxy diene was

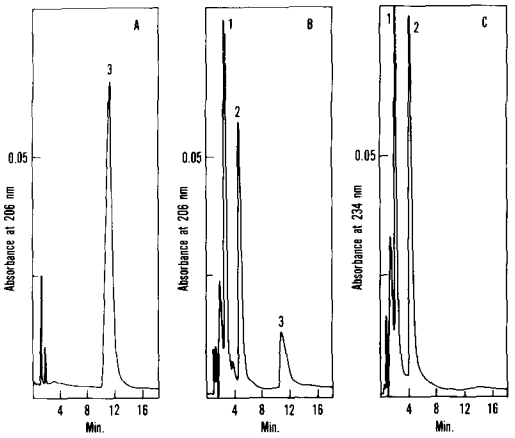

FIG. 2. Reverse-phase HPLC elution pattern of 20 μg di 18:2 PC resolved in 95% CH_3OH at 2 ml/min. A, 206 nm absorbance before oxidation; B, 206 nm absorbance after 50 hr oxidation; C, 234 nm absorbance after 50 hr oxidation.

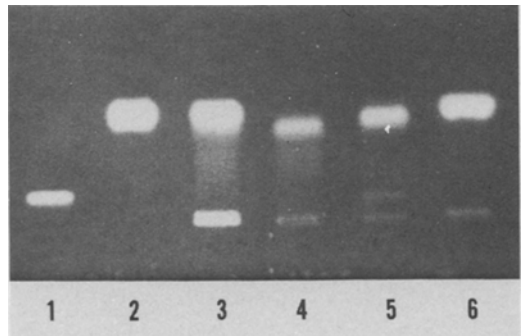

FIG. 3. Silica gel TLC of di 18:2 oxidation products resolved in $CHCl_3/CH_3OH/H_2O$ (65:35:4). Lane 1, lyso PC standard; lane 2, di 18:2 PC before oxidation; lane 3, total extract of di 18:2 PC oxidized 50 hr; lane 4, PC in Peak 1 (Fig. 2); lane 5, PC in Peak 2 (Fig. 2); lane 6, PC in Peak 3 (Fig. 2).

recovered from samples that had been reduced with $NaBH_4$, indicating that some of the fatty acids were hydroperoxides.

Although oxidized soy PC contains many forms of oxygenated fatty acid(s), hydroxy diene was the only product found in the aqueous autoxidation of di 18:2 PC. This implies that interaction between different molecular species can modify the oxidative products; results of studies on these interactions will be the subject of future reports.

ACKNOWLEDGMENTS

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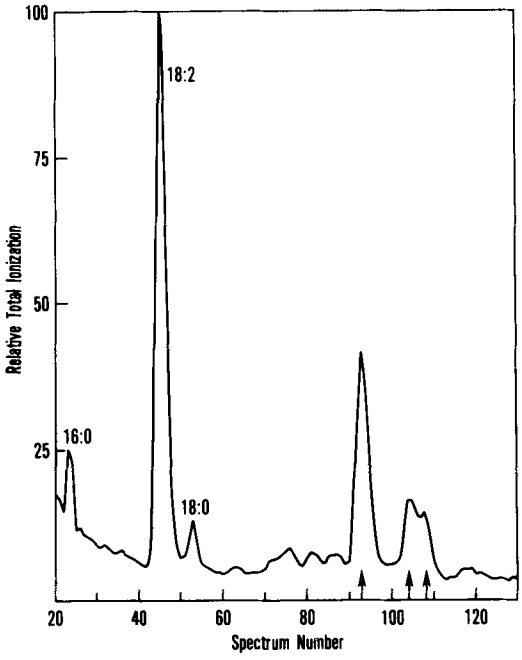

FIG. 4. GC-MS total ion chromatogram of the silylated methyl esters from the PC in Peak 2 (Fig. 2).

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Liver and Serum Lipids and Lipoproteins of Rats Fed 5% L-Lysine

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ABSTRACT

Soybean protein and casein supplemented with 1% Arg were compared for their ability to prevent fatty livers caused by excess dietary Lys. The concentrations of serum lipids and lipoproteins of rats fed 5% Lys and having fatty livers were also compared with those of rats fed the identical diet but lacking fatty livers when killed. The total liver lipids, triglycerides and cholesterol of rats fed 15% casein + 5% Lys were 3.9, 12.4 and 2 times control values, respectively. Rats fed 5% Lys + 1% Arg or 5% Lys with 15% soybean protein had liver lipid concentrations similar to controls fed no supplemental Lys. Serum total lipids, triglycerides, cholesterol, phospholipids and free fatty acids also did not change, and serum ketone bodies were slightly elevated with Lys feeding whether the rats had fatty livers or not. The concentrations of circulating HDL were slightly depressed in all rats fed 5% Lys while LDL were significantly elevated, particularly in rats without fatty livers. Serum VLDL did not change with 5% dietary Lys. Overall, excessive dietary Lys caused fatty livers which were prevented by varying the diet or length of feeding. Excess Lys feeding altered lipoprotein metabolism shown by decreased serum HDL and a substantial elevation in LDL. The latter was more apparent when the fat accumulation in liver was less severe or absent. The data suggest that the fatty liver from Lys excess is probably unrelated to increased fat mobilization from storage, decreased fat oxidation or to a major block in the transport of triglycerides from the liver to the circulation.

INTRODUCTION

Previously we reported that rats fed 5% L-lysine (Lys) with 15% casein for two weeks developed fatty livers which were not evident if 30% casein was fed (1). These fatty livers were attributed to the excessive intake of Lys and were not seen if 5% supplemental L-arginine (Arg), L-threonine, L-valine or L-glutamic acid was fed (2). The fatty livers of 5% Lys seen at two weeks were prevented by 1% supplemental Arg but only partially by 1% supplemental L-methionine and were absent after six weeks of 5% Lys feeding (2).

A feature common to fatty livers in rats from deficiencies of protein (3), Lys, threonine (4), methionine, choline (4,5) or Arg (6) or from toxic compounds (7,8) or orotic acid (9) is the accumulation of triglycerides which is accompanied by decreased concentrations of circulating lipids, particularly triglycerides and lipoproteins of low and very low density (3,5,7-11). Because a depression of plasma lipids and lipoproteins normally precedes the onset and then accompanies liver lipid accumulation, the majority of the fatty livers are believed to result from a major block in triglyceride transport from the liver to the circulation (7). The fatty liver of Lys, on the other hand, is associated with normal circulating total lipids, triglycerides, cholesterol and phospholipids (1,2).

The present studies compared the efficacy of Arg and soybean protein for preventing the fatty liver of excess Lys. They also compared the concentration of serum lipids and lipoproteins in rats fed Lys with or without fatty livers at time of killing. The production of fatty livers in rats was achieved by feeding 5% Lys with 15% casein for two weeks. Fatty livers were not seen if rats were fed 1% supplemental Arg, if soybean protein was fed instead of casein, or at the end of 6 weeks of 5% Lys feeding. Soybean protein was included in these comparisons because others had reported that supplementing soybean protein with excess Lys caused cholesterolemia without fatty livers in rabbits (12).

MATERIALS AND METHODS

Male weanling (30-50 g) Sprague-Dawley rats were caged individually in hanging wire cages in a room with a 12 hr dark-light cycle. All were fed ad libitum a 15% casein/soybean protein (1:1) diet similar to diet A in Table I until they were randomly assigned to the test diets as described below. Experiment I, a 2 x 3 factorial, employed 6 rats in each of the 6 treatments. There were three protein sources (casein, casein + 1% Arg and soybean protein alone) and two concentrations of supplemental Lys (0 and 5% Lys). Groups 1 and 3 were fed diets A and B in Table I, respectively. Groups 2 and 4 were fed, respectively, as 1 and 3, but 1% Arg was added at the expense of sucrose. Groups 5 and 6 were fed as 1 and 3 with soybean protein replacing casein. The experimental diets were fed for 14 days. The protocol for experiment II described in detail elsewhere (2) is briefly summarized here. It included two

groups of 5 rats each fed diets A and B for 42 instead of 14 days as in experiment I. Food intakes and body weights were recorded weekly. At the end of the feeding period, the rats were fasted for 4-6 hr and decapitated. Their blood was collected without anticoagulant, stored at 5 C for 24 hr and centrifuged at 3000 rpm (1500 x g) for 15 min to obtain serum. Approximately one gram of liver was homogenized in 9 ml water and lipids were extracted (13). Total liver lipids were determined gravimetrically while triglycerides (14), cholesterol (15) and phospholipids (16) were determined colorimetrically on the lipid extract. Cholesterol and triolein (Sigma Chemical Co., St. Louis, MO) were used as standards for the determination of cholesterol and triglycerides, respectively. A factor of 25 was used to convert phosphorus to phospholipids (17). Serum cholesterol (15), free fatty acids (18) and serum ketone bodies (19) were determined on the total serum. Palmitic acid and β-OH butyric acid (Sigma Chemical Co., St. Louis, MO) were used as standards for the determination of free fatty acids and ketone bodies, respectively. Serum lipoproteins were stained and separated by polyacrylamide gel electrophoresis as described by Naito et al. (20). The proportions of stainable pre β, β and α, corresponding to very low, low and high density lipoprotein, respectively, were determined by scanning at 580 nm and integration. Total serum lipids were determined gravimetrically and serum triglycerides (14) and phospholipids (16) colorimetrically after lipid extraction (21). The data in experiment I were analyzed by factorial analysis of variance (22), and means were compared by Duncan's multiple range test (23). The data in experiment II were analyzed by t-test (22). In experiment II, only serum lipoproteins are reported because growth, food intakes and serum and liver lipids have been described previously (2).

TABLE I

Basal Diet Composition

	Percent	
	A	B
Protein[a]	15	15
Sucrose[b]	73.7	68.7
Corn oil[c]	5	5
Vitamin[d]	1	1
Minerals[e]	5	5
L-Methionine[f]	0.3	0.3
L-Lysine · HCl[f]	0	5

[a]Vitamin-free test casein or soya assay protein, both from Teklad (Madison, WI) with catalog No. 160040 and 160480, respectively.

[b]Amalgamated Sugar Co., Ogden, UT.

[c]Pure corn oil, Anderson Clayton Foods, Dallas, TX.

[d]Vitamin Fortification Mix, Teklad, Catalog No. 40060.

[e]Rogers-Harper Mineral Mix, Teklad, Catalog No. 170760.

[f]Pyrogen-free injectable grade.

RESULTS

The respective concentrations of liver lipids, triglycerides and cholesterol in rats fed 5% Lys with casein in experiment I were 3.9, 12.4 and 2 times control values, respectively, from rats fed no supplemental Lys (Groups 1 and 3 in Table II). The concentrations of these classes of lipids in livers of rats fed 5% Lys with casein + 1% Arg or with soybean protein instead of casein were normal. The pooled mean values showed that rats fed 5% Lys consumed 1.5 g or 10% less food per day, gained 1.3 g or 22% less in body weight and were 14% less efficient in utilizing food for weight gain. Rats fed 5% Lys + 1% Arg gained only 9% less than controls, whereas rats fed 5% Lys with soybean protein gained 18% less. Serum total lipids, triglycerides, cholesterol, phospholipids and free fatty acids remained unchanged with 5% Lys feeding. Serum ketones were elevated in all rats fed 5% Lys. The concentration of serum HDL was slightly below control for rats fed 5% Lys when analyzed by factorial analysis of variance ($P < .012$, Table II). The concentration of serum LDL increased with 5% Lys feeding, particularly in rats fed 5% Lys with soybean protein. Rats fed casein + 5% Lys showed a wide range of total liver lipids (6.6 to 28.9 mg/100 mg liver) and serum LDL (4.2 to 28.6%). Regression analysis within this group showed that the concentration of serum LDL was inversely related to total liver lipids (Fig. 1). The concentration of VLDL did not respond to Lys feeding, but it was substantially lower in the groups fed soybean protein.

Rats of experiment II fed Lys for 6 weeks had concentrations of liver and serum triglycerides, cholesterol and phospholipids which were identical to controls. These data have been reported elsewhere (2), but 5% Lys caused a depression in the concentration of serum HDL (Fig. 2) and a 2.9-fold increase in serum LDL (Figs. 2,3). The HDL/LDL ratio (Fig. 3) also decreased from 19.8 to 6.2 with 5% Lys feeding, but the concentration of serum VLDL did not change. All lipoprotein concentrations

TABLE II

Food Intake, Growth, Feed Efficiency, Liver Weight, Liver (mg/100 mg) and Serum (mg/100 ml) Lipids and % Serum Lipoproteins for Male Sprague-Dawley Rats Fed 0 or 5% L-Lysine with 15% Casein, 15% Soybean Protein or with 15% Casein + 1% L-Arginine[1]

Group	1	2	3	4	5	6	p[3]		
Protein source	Casein	Casein + Arg	Casein	Casein + Arg	Soybean	Soybean			
L-lysine (%)	0	0	5	5	0	5	Lys	Ptn	Lys x Ptn
Food intake (g/day)	15.7 ± 0.3^{a}	15.6 ± 0.7^{b}	13.7 ± 0.7^{b}	14.4 ± 0.4^{ab}	13.3 ± 0.2^{bc}	12.1 ± 0.5^{c}	.001	.001	.528
Growth (g/day)	6.5 ± 0.2^{a}	6.6 ± 0.2^{a}	4.2 ± 0.2^{cd}	5.9 ± 0.2^{b}	4.4 ± 0.1^{c}	3.6 ± 0.4^{d}	.001	.001	.006
Feed efficiency %	42 ± 1^{a}	43 ± 1^{a}	31 ± 2^{b}	41 ± 0.5^{ab}	33 ± 1^{bc}	29 ± 2^{c}	.001	.001	.528
Liver weight	9.1 ± 0.3^{a}	8.9 ± 0.2^{a}	8.3 ± 0.4^{a}	8.4 ± 0.5^{a}	6.7 ± 0.2^{b}	5.0 ± 0.6^{c}	.003	.001	.258
Total liver lipids	4.0 ± 0.2^{a}	4.7 ± 0.2^{a}	15.6 ± 3.4^{b}	5.5 ± 0.4^{a}	5.4 ± 0.3^{a}	4.9 ± 0.3^{a}	.002	.002	.001
Liver triglycerides	0.92 ± 0.04^{a}	1.0 ± 0.13^{a}	11.4 ± 3.8^{b}	1.50 ± 0.28^{a}	1.20 ± 0.21^{a}	0.86 ± 0.19^{a}	.001	.004	.001
Liver cholesterol	0.24 ± 0.01^{a}	0.25 ± 0.01^{a}	0.48 ± 0.07^{b}	0.27 ± 0.01^{a}	0.28 ± 0.01^{a}	0.29 ± 0.02^{a}	.010	.002	.002
Liver phospholipids	1.51 ± 0.09^{a}	1.68 ± 0.06^{ab}	1.85 ± 0.08^{bc}	1.70 ± 0.11^{ab}	1.85 ± 0.07^{bc}	2.03 ± 0.05^{c}	.010	.003	.181
Total serum lipids	423 ± 26^{ab}	430 ± 33^{ab}	390 ± 42^{b}	503 ± 11^{a}	377 ± 29^{b}	347 ± 30^{b}	.914	.005	.153
Serum triglycerides	115 ± 12^{ab}	115 ± 7^{ab}	114 ± 18^{ab}	128 ± 14^{a}	88 ± 7^{bc}	69 ± 9^{c}	.818	.002	.397
Serum cholesterol	99 ± 5	100 ± 4	99 ± 8	99 ± 7	89 ± 10	69 ± 3	.963	.181	.999
Serum phospholipids	146 ± 8^{ab}	158 ± 14^{ab}	139 ± 13^{b}	175 ± 10^{a}	146 ± 6^{ab}	138 ± 12^{b}	.933	.054	.461
Serum free fatty acids	29.5 ± 2.2^{ab}	31.7 ± 2.2^{ab}	29.9 ± 2.5^{ab}	34.9 ± 1.9^{a}	29.4 ± 1.8^{ab}	26.8 ± 2.0^{b}	.877	.049	.392
Serum ketone bodies	5.7 ± 0.09^{a}	5.1 ± 0.13^{a}	7.0 ± 0.7^{ab}	6.3 ± 0.5^{ab}	6.0 ± 0.3^{ab}	7.8 ± 1.0^{b}	.009	.161	.885
Serum HDL %[2]	84.7 ± 1.8	87.3 ± 0.6	81.2 ± 4.0	82.5 ± 2.8	88.2 ± 1.1	82.4 ± 1.9	.012	.779	.976
Serum LDL %[2]	8.8 ± 1.6^{a}	8.0 ± 0.7^{a}	12.9 ± 3.7^{ab}	12.4 ± 2.1^{ab}	9.4 ± 0.9^{ab}	16.2 ± 2.0^{c}	.005	.490	.063
Serum VLDL %[2]	6.8 ± 0.9^{b}	4.7 ± 0.8^{ab}	5.9 ± 1.0^{b}	5.2 ± 1.2^{b}	2.4 ± 0.3^{a}	2.4 ± 0.5^{a}	.906	.001	.743
Serum HDL/LDL	11.4 ± 2.0	10.9 ± 1.0	6.3 ± 2.8	6.7 ± 1.4	9.4 ± 1.1	5.1 ± 0.9	.131	.233	.433
Number of rats	6	6	6	6	6	7			

[1]Rats were fed the diets described in the method section for Experiment I for 2 wks. Entries are means ± standard error of the mean. [a-c]Horizontal values not sharing a common superscript are different ($P < 0.05$) according to Duncan's multiple range test.

[2]Percent of the total area under the HDL, LDL and VLDL peaks.

[3]P represents the probability for an effect of lysine (Lys), protein source (Ptn) or their interactions (Lys x Ptn).

were determined by lipid staining methods (20).

DISCUSSION

The present data show that replacing casein with soybean protein was as effective as supplemental Arg in preventing the fatty liver of excess Lys. Because fatty livers of excess Lys can be prevented by supplemental Arg, it has been suggested that excess Lys causes an Arg deficiency (2, Ulman, Kari, Hevia and Visek, unpublished data) in agreement with Lys-Arg antagonism which is well known (24), and with the orotic aciduria which accompanies Arg deficiency or excess Lys feeding (2). Orotic aciduria is a sign of Arg deficiency (25). The prevention of the fatty liver of Lys excess by soybean protein reported here also agrees with this hypothesis because soybean protein is richer in Arg with respect to Lys and contains approximately twice as much Arg as casein (26). Serum total lipids, triglycerides and phospholipids in experiment I as in experiments reported previously (1,2) were the same in control rats as in rats fed Lys with or without fatty livers. Such similar patterns have not been seen with fatty livers caused by other means (1,3-11).

These experiments also showed that serum free fatty acids were similar in controls and in rats fed 5% Lys whether they had fatty livers or not. Because fatty livers arising from enhanced fat mobilization from adipose tissue are associated with elevated circulating free fatty acids (27-29), the present data argue that fatty livers of Lys excess arise by other mechanisms. Serum ketone bodies, on the other hand, were elevated by 5% supplemental Lys but to a similar extent whether the rats had fatty livers or not. This suggests that altered ketone body production likewise did not cause these fatty livers.

Five percent dietary Lys reduced the concentration of circulating high density lipoproteins. Factorial analysis of variance revealed this reduction in experiment I and indicated that HDL was similarly reduced in all rats fed Lys regardless of the fat content of their liver. A reduction in HDL was also observed in rats fed Lys without fatty livers in experiment II. The concentration of serum low density lipoproteins, however, was higher with Lys, particu-

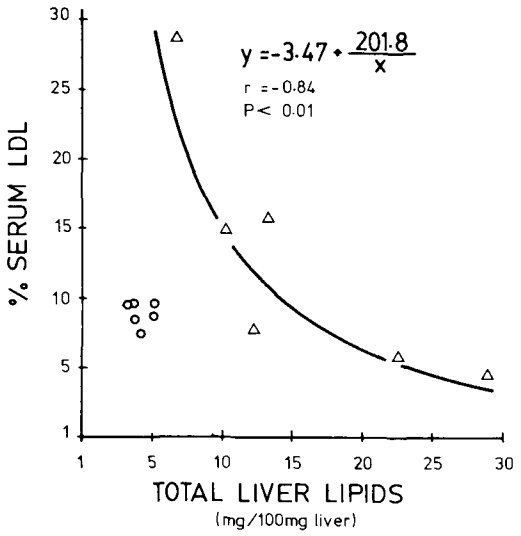

FIG. 1. The inverse relationship between the concentration of serum LDL and total liver lipids for the six rats fed 15% casein + 5% L-Lys (△) and 15% casein (○) in Experiment I. r = regression coefficient.

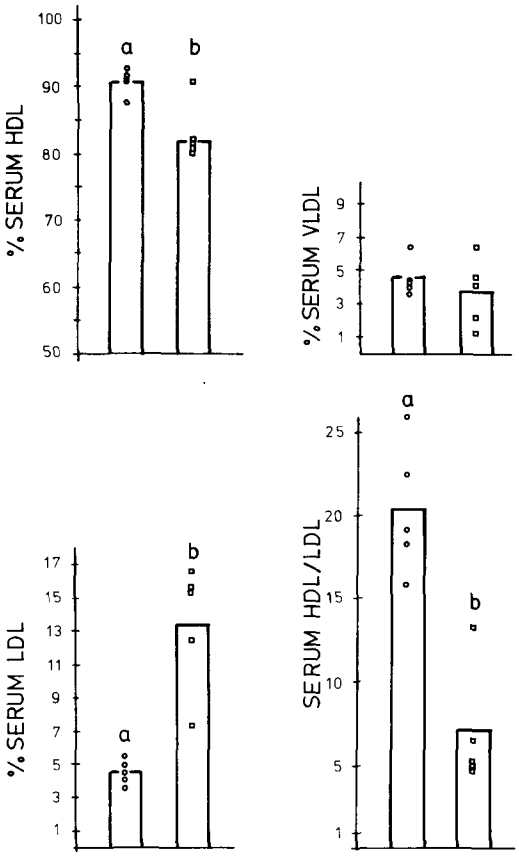

FIG. 2. Serum concentrations (%) of HDL, LDL, VLDL and HDL/LDL in rats fed 15% casein (○) and 15% casein + 5% L-Lys (□) for 6 weeks in Experiment II. Rats fed 5% L-Lys for 6 weeks had triglycerides, cholesterol, and phospholipids in serum and liver which did not differ from control rats (2). $^{a\text{-}b}$Rats showing a different superscript differ at 5% level by Students t test.

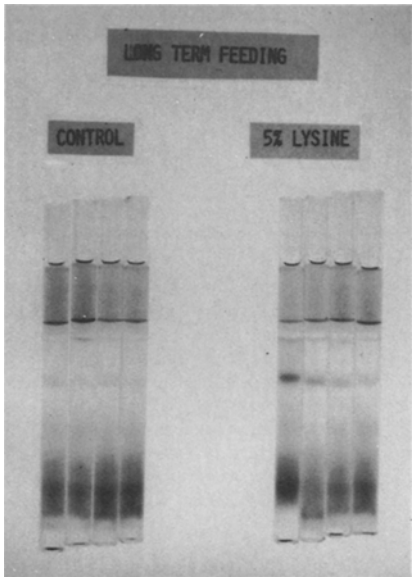

FIG. 3. Polyacrylamide gel electrophoresis patterns of Sudan Black prestained serum for 4 of the 5 rats fed either 15% casein (controls) or 15% casein + 5% L-Lys (5% lysine) for 6 weeks. Rats fed 5% L-Lys for 6 weeks (long term feeding), in contrast to those fed 5% L-Lys for 2 weeks, did not have fatty livers (2). The lipoprotein distribution from bottom to top is HDL, LDL, VLDL and chylomicrons.

larly in rats without fatty livers. The effect of the concentration of liver lipids upon the elevation of LDL from excessive dietary Lys was apparent in experiment I, with all rats fed Lys having higher LDL than controls and those fed Lys with soybean protein having the highest but no fatty livers. Similarly, in experiment II, rats with fatty livers from excess Lys no longer evident after 6 weeks had LDL concentrations which were 2.9 times controls. Finally, the inverse relationship between serum LDL and total liver lipids (Fig. 1) revealed by regressing the concentration of serum LDL against the concentration of total liver lipids of the six rats fed 5% Lys with fatty livers in experiment I (group 3 in Table II) confirmed the previous observation by showing that within this group high LDL were seen only when liver lipids approximated control values. In contrast to the decrease in HDL and the increase in LDL with 5% Lys feeding, the concentration of serum VLDL did not change. The lowest concentration of serum VLDL was seen in rats fed soybean protein.

The failure to demonstrate a substantial decline in circulating lipids and lipoproteins of low densities in rats fed 5% Lys with fatty livers strongly suggests that, in contrast to fatty livers produced by other means (3,5,7-11), fatty livers from excess Lys did not result from a major block in the transport of lipids from the liver to the circulation.

The data show that excessive dietary Lys not only causes a fatty accumulation in the liver of rats which is prevented or abolished by dietary manipulation, but it also causes changes in circulating lipoproteins as measured by lipid staining (20), particularly an elevation in LDL which becomes more apparent when the fatty liver has been prevented by the diet or has disappeared during a longer period of excess Lys feeding.

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The Effect of Isomeric *trans*-18:1 Acids on the Desaturation of Palmitic, Linoleic and Eicosa-8,11,14-trienoic Acids by Rat Liver Microsomes

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ABSTRACT

The inhibitory effects of the positional isomers of *trans*-18:1 acids on the desaturation of palmitic acid to palmitoleic (Δ9-desaturase), linoleic to γ-linolenic (Δ6-desaturase) and eicosa-8,11,14-trienoic to arachidonic acid (Δ5-desaturase) were investigated. These *trans*-18:1 acids were found to be inhibitory for the microsomal Δ6-, Δ9- and Δ5-desaturases of rat liver. The position of the double bond in the *trans*-18:1 acids seems to be important in determining the degree of inhibition. At inhibitor/substrate ratio of 3:1, the Δ6-desaturase was most strongly inhibited by *trans*-Δ3, -Δ4, -Δ7 and -Δ15-18:1 isomers, whereas the Δ9-desaturase was most strongly inhibited by *trans*-Δ3, -Δ5, -Δ7, -Δ10, -Δ12, -Δ13 and -Δ16 isomers. At inhibitor/substrate ratio of 6:1, the Δ5-desaturase was most strongly inhibited by Δ3-, Δ9-, Δ13- and Δ15-isomers. When 18:0 was added to the incubations of 16:0, 18:2 and 20:3 at the same I/S ratios used for the *trans*-18:1 acids, weak inhibition for Δ9-desaturase and no inhibition for Δ5- and Δ6-desaturases was observed.

INTRODUCTION

The inhibition of the metabolism of essential fatty acids by other unsaturated fatty acids was demonstrated in vivo by Mohrhauer and Holman (1), and in vitro by Brenner et al. (2). Since then, many investigations on the effect of unsaturated fatty acids upon the desaturation of various fatty acids in vitro have been conducted (3). The existence of competitive reactions among oleic, linoleic and linolenic acids was demonstrated (2,4). Such widely different unsaturated acids as docosahexaenoic acid (5), petroselinic, vaccenic, elaidic and also *trans*-linoleic acid caused an inhibition of the desaturation of linoleic acid to γ-linolenic acid (6). Chang et al. (3) found that octadecenoic and octadecynoic acids inhibit the desaturation of stearic acid to oleic acid and that the degree of inhibition greatly depends on the position of the double or triple bond. Fatty acids of different chain lengths and numbers of double bonds belonging to the oleic, linoleic and linolenic acids series have inhibitory action on the desaturation of eicosa-8,11-dienoic acid to eicosa-5,8,11-trienoic acid (7). For a given chain length, the increasing number of double bonds increased the degree of inhibition, but the position of the double bond in the fatty acid molecule also influenced the degree of inhibition.

Trans isomers of essential fatty acids (EFA) are unable to prevent development of EFA deficiency (8-11) and may increase the animal's requirement for EFA (9,11,12). In this laboratory, Hill et al. (13) showed that ingestion of margarine stock (partially hydrogenated soybean oil containing 45.8% total *trans* fatty acids or 43% as *trans*-18:1) intensified the EFA deficiency. The growth and the dermal scores of rats fed margarine stock indicated a more severe EFA deficiency than in the rats fed an equal level of saturated fat. These nutritional studies used hydrogenated fats which contain mixtures of many *trans* fatty acids principally isomers of 18:1. The present study investigated the effects in vitro of the individual positional isomers of *trans*-18:1 acids on the desaturation of palmitic acid to palmitoleic acid (Δ9-desaturase), linoleic to linolenic (Δ6-desaturase) and eicosa-8,11,14-trienoic to arachidonic acid (Δ5-desaturase) by rat liver microsomes from EFA deficient rats.

MATERIALS AND METHODS

Reagents and Substrates

ATP, CoA, NADH and bovine serum albumin V fraction were obtained from Sigma Chemical Co., St. Louis, MO. Palmitic acid, linoleic acid, and eicosa-8,11,14-trienoic acid were obtained from NuChek Prep, Elysian, MN. Palmitoyl-CoA was obtained from Sigma. [1-^{14}C] Palmitic acid (sp. act. 56 mCi/mmole), [1-^{14}C] linoleic acid (sp. act. 51 mCi/mmole) were purchased from Amersham Corp., Arlington Heights, IL. [1-^{14}C] Eicosa-8,11,14-trienoic acid (sp. act. 55 mCi/mmole) and [1-^{14}C]-palmitoyl-CoA (sp. act. 53.5 mCi/mmole) were purchased from New England Nuclear, Boston, MA.

Synthesis of Linoleoyl-CoA

Linoleoyl-CoA was synthesized from a mixture of labeled and unlabeled linoleic acid by the procedure described by Reitz et al. (14).

The precipitated linoleoyl-CoA was then purified (15) under an atmosphere of nitrogen, and the final preparation was kept in aqueous solution, pH 5.0, under nitrogen at -20 C. The yield was ca. 4 μmoles of linoleoyl-CoA per 10 μmoles of CoA used as starting material. The concentrations of linoleoyl-CoA determined by the UV spectrophotometric (16) and measurement of phosphate (17) were consistent. The amount of CoA released by the acyltransferase system (18) showed that the synthesized material was 100% reactive. Gas liquid chromatography was performed on the methyl ester, obtained by heating of the acyl-CoA with 0.5 N alcoholic NaOH under N_2 and methylation of the liberated fatty acids with HCl in methanol, as an additional check on the purity. It gave one symmetrical peak for 18:2 acid.

Preparation of Microsomes

Weanling male Sprague-Dawley rats kept on EFA deficient diets for at least 6 months were sacrificed and the livers were quickly removed and rinsed in cold homogenization solution consisting of 0.15 M KCl, 0.005 M $MgCl_2$, 0.004 M EDTA, 0.25 M sucrose, 0.0015 M glutathione, 0.05 M potassium phosphate buffer (pH 7.4) (19). They were then homogenized in a Teflon pestle homogenizer in 3 volumes of the same solution. The homogenated was centrifuged at 12,000 x g for 30 min. The microsomes were recovered by centrifuging the supernatant at 140,000 x g for 2 hr in a Beckman L5-75 ultracentrifuge. The pellets were resuspended in the homogenization solution to provide microsomes equivalent to 1 g of original liver in one ml. The mixture was stirred, put into screw-capped tubes under nitrogen, quickly frozen and stored at -70 C. Protein was determined by the biuret reaction. All operations were carried out at 4 C.

Incubation Conditions

Each incubation contained the following: 7.5 μmole of ATP, 2.6 μmole of NADH, 1 μmole of CoA, 7.5 μmole of $MgCl_2$, 100 μmole of potassium phosphate buffer (pH 7.4), 0.15 M KCl-0.25 M sucrose, 2 mg microsomal protein (except as stated) and the desired fatty acid(s) in a total volume of 1.0 ml. The incubations were carried out for 20 min in a Dubnoff metabolic shaker at 37 C. The fatty acids were used as sodium salt-albumin complex (1 μg free fatty acid/11.55 μg bovine serum albumin).

Analysis of Fatty Acids

The reaction was terminated by the addition of 5% HCl in methanol, and the lipids were extracted with chloroform/methanol (2:1, v/v). The extract was dried under a stream of nitrogen and esterified with 10% HCl in methanol at 80 C for 2 hr. The tubes were evaporated to dryness under nitrogen, the esters redissolved in petroleum ether, and a mixture of unlabeled carriers of methyl esters of 16:0 + 16:1, 18:2 + 18:3 or 20:3 + 20:4 was added to the incubation products. The esters were separated on 10% $AgNO_3$ Silica Gel H plates in petroleum ether/diethyl ether (95:5, v/v) or chloroform/methanol (100:2, v/v) or petroleum ether/diethyl ether/acetic acid (70:3:1, v/v) for the separation of the substrate and products of 16:0, 18:2 and 20:3 acid incubations, respectively. The separated bands were made visible under UV light after spraying with 0.1% 2,7-dichlorofluorescein. The bands were scraped into scintillation vials, 15 ml of toluene-based scintaillation fluid was added and the activity was determined in a Packard scintillation counter. The percent desaturation was calculated as the ratio of the counts in the desaturated product to the sum of the counts in the substrate plus product. The nmoles of the product were then calculated. The recovered radioactivity was more than 90% of the amount used.

RESULTS AND DISCUSSION

Substrate saturation curves were measured for all substrates that were to be used to study the desaturation reaction (20) because free fatty acids inhibit a variety of enzymatic reactions (21). In competitive experiments, as described subsequently, it was necessary to establish that when inhibition was observed, it was due to some type of interaction other than substrate inhibition (20). In the microsomal desaturation studies, both substrates and products are incorporated into microsomal lipids (22-24). In the experiments reported here, even though substrate and product were incorporated into complex lipids, the use of substrate levels that give maximum desaturation should minimize the effects of incorporation into complex lipids upon the rate of desaturation (6).

The substrate saturation curves for the desaturation of 18:2, 20:3 and 16:0 were studied. After 20 min incubation period when the 16:0 level was 100 nmoles, the amount of 16:1 formed was 45 nmoles (45% conversion); when the 18:2 level was 100 nmoles, the amount of 18:3 formed was 16 nmoles (16% conversion); and when the amount of 20:3 was 20 nmoles, the amount of 20:4 was 10 nmoles (50% conversion). In the inhibition study, 100

nmole of 16:0 or 18:2 and 20 nmoles of 20:3 were used because at these substrate concentrations, the highest percent conversions were obtained.

Simultaneous incubations of 100 nmoles of 16:0, 100 nmoles of 18:2 or 20 nmoles of 20:3 acid substrates in the presence of the *trans*-18:1 acid inhibitors were made to cover a range of inhibitor/substrate (I/S) ratios from 0.25 to 10 (Fig. 1). Figure 1 shows the relation between the percent inhibition and the concentration of the inhibitor acids added. The *trans*-18:1 isomers were more effective as inhibitors (throughout the I/S ratio range of 0.25 to 10)

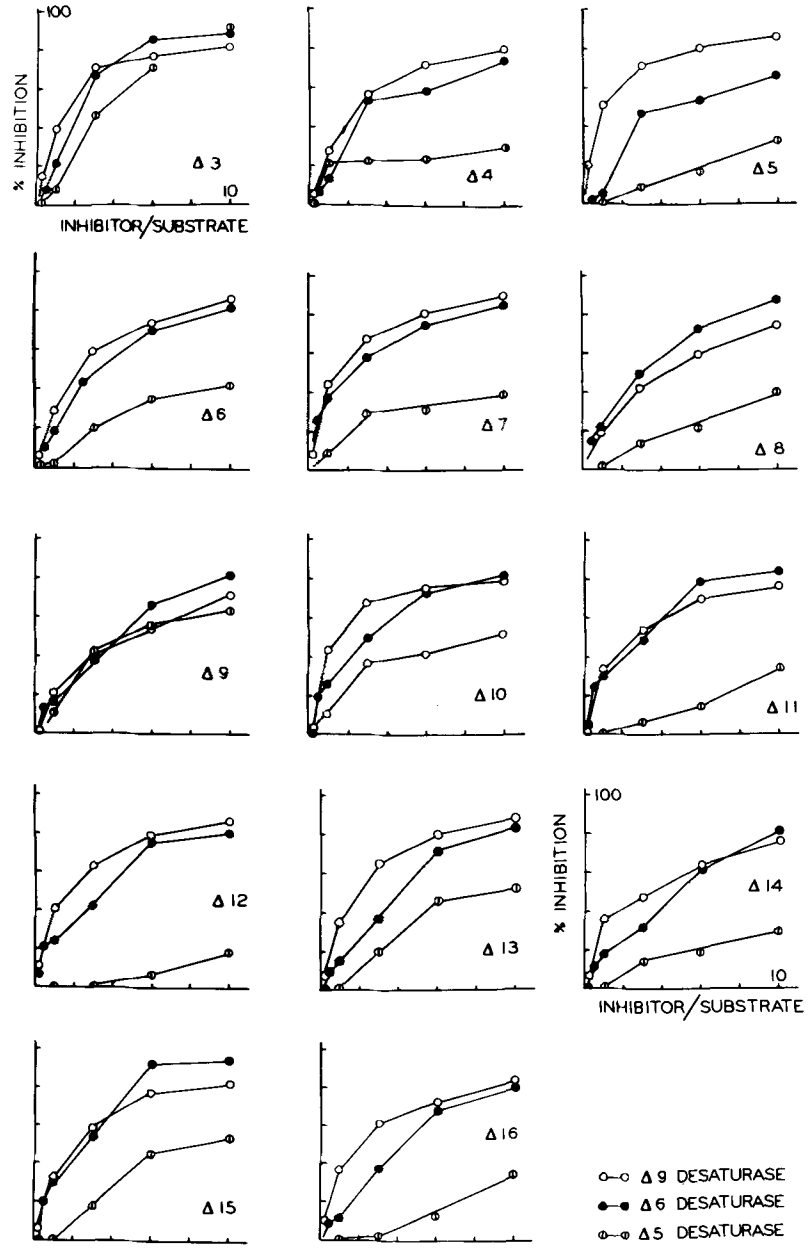

FIG. 1. The effect of increasing I/S ratio on the percent inhibition of the desaturation of palmitic, linoleic and eicosa-8,11,14-trienoic acids. The incubation mixture contained 100 nmoles of either 16:0 or 18:2 or 20 nmoles of 20:3ω6 as substrates with increasing concentration of isomeric *trans*-octadecenoic acids as inhibitors.

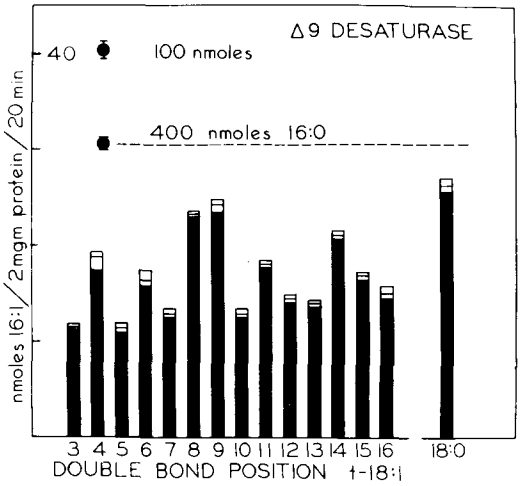

FIG. 2. The effect of the position of the double bond in isomeric *trans*-octadecenoic acids and stearic acid upon the desaturation of [1-^{14}C] palmitic acid. All inhibitors were present in a concentration of 300 nmoles with 100 nmoles of palmitic acid as substrate (I/S, 3:1). Control values for the desaturation of 100 or 400 nmoles of palmitic acid with no inhibitors are shown.

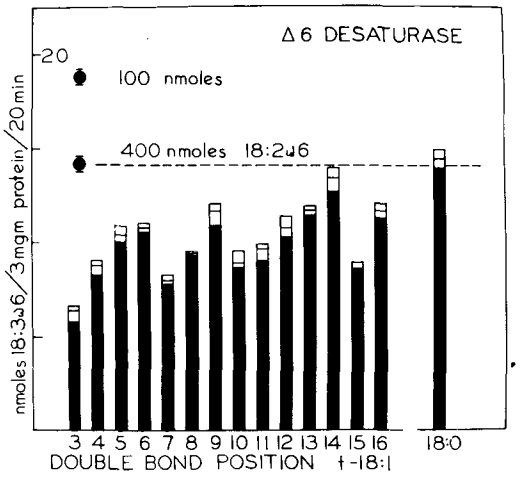

FIG. 3. The effect of the position of the double bond in isomeric *trans*-octadecenoic acids and stearic acid upon the desaturation of [1-^{14}C] linoleic acid. All inhibitors were present in a concentration of 300 nmoles with 100 nmoles of linoleic acid as substrate (I/S, 3:1). Control values for the desaturation of 100 or 400 nmoles of linoleic acid with no inhibitors are shown.

on the desaturation of 16:0 → 16:1 (Δ9 desaturase) and 18:2 → 18:3 (Δ6 desaturase) than on the desaturation of 20:3 → 20:4 (Δ5 desaturase).

As the concentration of the inhibitor acids was increased, the inhibition of the desaturation of 16:0 → 16:1 and 18:2 → 18:3 was increased to ca. 80% when the I/S ratio reached 10. All the *trans* acids except Δ8 and Δ9 inhibited the desaturation of 16:0 → 16:1 more than the desaturation of 18:2 → 18:3 as the I/S ratio increased from 0.25 to 4.0. The desaturation of 20:3 → 20:4 was maximally inhibited (20 to 50%) at the inhibitor/substrate ratio of 10, except in the cases of the Δ3 and Δ9 isomers which gave ca. 85 and 60% inhibition, respectively.

To compare more directly the effect of the double bond position, desaturation was measured with each *trans*-18:1 isomer at I/S ratio 3:1 in case of Δ9- and Δ6-desaturase and 6:1 for the Δ5-desaturase. These are the I/S ratios at which some of the *trans*-18:1 isomers gave ca. 50% inhibition. In these experiments, the presence of inhibitor concentration 3 or 6 times that of the substrate increased the amount of potential fatty acid substrate in the system 4 times in case of 16:0 and 18:2 or 7 times in case of 20:3. Therefore, comparison of inhibitions was made with a control in which the total substrate concentration was 4 or 7 times the concentration of labeled substrate to be used. These values are shown in Figures 2, 3 and 4.

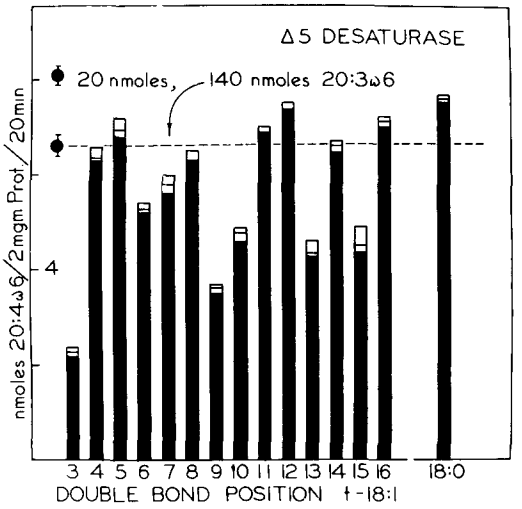

FIG. 4. The effect of the position of the double bond in isomeric *trans*-octadecenoic acids and stearic acid upon the desaturation of [1-^{14}C] eicosa-8,11,14-trienoic acid. All inhibitors were present at a concentration of 120 nmoles with 20 nmoles of 20:3 as substrate (I/S, 6:1). Control values for the desaturation of 20 or 140 nmoles of 20:3ω6 with no inhibitors are shown.

Figure 2 shows that *trans*-Δ3, -Δ5, -Δ7, -Δ10, -Δ12, -Δ13 and -Δ16 were the most effective inhibitors of the desaturation of 16:0, whereas Δ8 and Δ9 were the least effective

inhibitors. Figure 3 shows that *trans*-Δ3 and -Δ7 18:1 were the most effective inhibitors for the desaturation of 18:2. For the desaturation of 20:3 → 20:4, the *trans* isomers Δ3, Δ9, Δ13 and Δ15 were the most effective inhibitors (Fig. 4), whereas the *trans*-Δ4, Δ5, Δ8, Δ11, Δ12, Δ14 and Δ16 were not effective as inhibitors.

Our data agree with those obtained by Brenner and Peluffo (6) in that *trans*-Δ11 is more effective than *trans*-Δ9 as inhibitor for the desaturation of linoleic acid, but the degree of inhibition obtained in our study is somewhat higher. This may be due to the difference in the relative proportion of fatty acids to the microsomal protein which has some effect (6).

To examine the type of inhibition produced by the *trans* isomers on the desaturation reactions of 16:0, 18:2 and 20:3 acids, three different positional isomers that caused inhibition were studied in more detail for each desaturation reaction. Although the substrate and products are incorporated into microsomal lipids (4,22,24), and Lineweaver-Burk plots do not strictly apply, these plots (Figs. 5A, 6A and 7) showed convergent lines which intersected at the ordinate when increasing amounts of each substrate (20, 40, 60, 80 and 100 nmoles) were incubated for 5 min in the absence or presence of a constant level of the *trans* isomers (100 nmoles). These data suggest that the inhibition is competitive.

Apparent K_m and K_i values were calculated from the graphs and are shown in Table I. In a heterogeneous reaction system such as ours, the

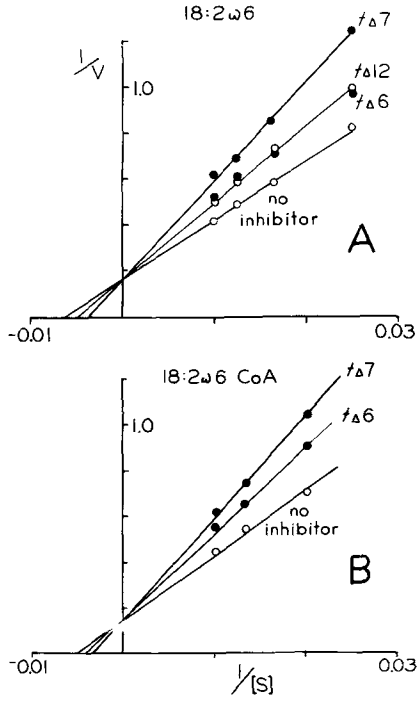

FIG. 5. Lineweaver-Burk plots for [1-^{14}C] linoleic acid (A) and linoleoyl-1-^{14}C-CoA (B) incubations, in the absence or presence of 0.1 mM of *trans*-Δ^6, Δ^7, and Δ^{12}-18:1 acids. Velocities were measured for the first 5 min of incubation at 37 C under the conditions described in the text. Each tube contained 3 mg microsomal protein of EFA-deficient rat livers.

TABLE I

Apparent Michaelis Constants for Oxidative Dehydrogenations of Palmitic, Linoleic and Eicosatrienoic Acids[a]

Labeled substrate incubated	K_m for substrate	*Trans*-18:1 isomers as inhibitors	K_i
Palmitic acid	6.67 x 10^{-5} M	t Δ^5	4.3 x 10^{-5} M
		t Δ^9	45 x 10^{-5} M
		t Δ^{12}	11.42 x 10^{-5} M
Palmitoyl-CoA	6.7 x 10^{-5} M	t Δ^5	5 x 10^{-5} M
		t Δ^9	33 x 10^{-5} M
		t Δ^{12}	10 x 10^{-5} M
Linoleic acid	16 x 10^{-5} M	t Δ^6	40 x 10^{-5} M
		t Δ^7	14.8 x 10^{-5} M
		t Δ^{12}	40 x 10^{-5} M
Linoleoyl-CoA	20 x 10^{-5} M	t Δ^6	40 x 10^{-5} M
		t Δ^7	15 x 10^{-5} M
Eicosatrienoic (8,11,14) acid	2.5 x 10^{-5} M	t Δ^{10}	3.8 x 10^{-5} M
		t Δ^{13}	11 x 10^{-5} M

[a]The K_m values were calculated from Lineweaver-Burk plots. The velocities were measured for the first 5 min of incubations of the labeled acid with 2 mg of microsomal protein (3 mg in case of 18:2) at 37 C. K_i values were also calculated from the graphs.

significance of the absolute values of these parameters is unclear and these values do not measure the affinity of the enzyme for the substrate. Classically substrate saturation and competitive inhibition have been related to the direct interaction of enzyme and substrate or inhibitor. Alternatively, for reactions involving an acyl-CoA and a membrane-bound enzyme, saturation behavior may reflect the interaction of substrate with the membrane-water interface (25). In any case, comparison of the constants obtained with fatty acid vs. fatty acyl-CoA as substrate could be used to show that the activation step of the fatty acid is not a limiting step reaction in the system. The rates measured in our enzymatic systms are the result of several reactions, including acylation of the substrate with CoA, desaturation and esterification of sbustrate and products (4). The slowest rate will limit the overall rate of the reaction. There is sufficient NADH and CoA in our system to eliminate these cofactors as limiting factors. This also was definitely proven when the competition of linoleoyl-CoA (Fig. 5B) and palmitoyl-CoA (Fig. 6B) was tested and convergent lines intersecting at the ordinate were obtained.

Our study on the desaturation of isomeric *trans*-18:1 acids by rat liver microsomes showed that all the 18:2 acids from the desaturation of *trans*-18:1 acids contained a *cis*-Δ9 double bond indicating the action of Δ9-desaturase (26). Thus, the *trans*-18:1 acids act as alternative substrates with 16:0 for Δ9-desaturase. This can be seen in Figure 2 where the *trans*-Δ8- and -Δ9-18:1 acids, which were not desaturated, were the weakest inhibitors, and the levels of desaturation of some of the *trans*-18:1 acids (Δ5, 23.9%; Δ6, 14.1%; Δ8, 0.58%; Δ9, 1.2%; Δ13, 17.2%; and Δ14, 10.5%) paralleled their inhibitory effects. On the other hand, some of the other isomers which were not desaturated (Δ10) or desaturated at low level (Δ7, 3.9% and Δ15, 3.2%) caused significant inhibition, indicating another possible mechanism for the inhibitory action of *trans*-18:1 acids. Moreover, stearic acid which also acts as alternative substrate for Δ9-desaturase, when added to the incubation of 16:0 at the same I/S ratio used for the *trans*-18:1 acids, showed a relatively weak inhibition compared to the *trans*-18:1 acids (Fig. 2), although the level of desaturation of 18:0 by Δ9-desaturase is higher than any of the *trans*-18:1 acids. This also indicates that *trans*-18:1 acids may inhibit by another mechanism, perhaps through interaction with the membrane-water interface (25) as discussed above. When 18:0 was added to the incubations of 18:2 and 20:3 at the same I/S

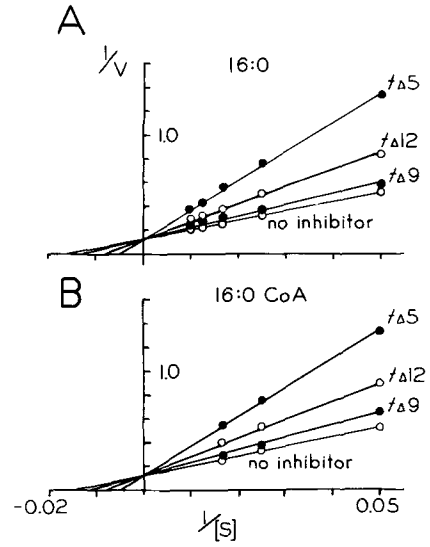

FIG. 6. Lineweaver-Burk plots for [1-^{14}C] palmitic acid (A) and palmitoyl [1-^{14}C] CoA (B) incubations, in the absence or presence of 0.1 mM of *trans*-Δ^5, Δ^9 and Δ^{12}-18:1 acids. Velocities were measured for the first 5 min of incubation at 37 C under the conditions described in the text. Each tube contained 2 mg microsomal protein of EFA-deficient rat livers.

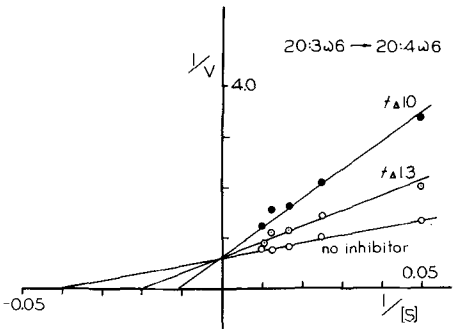

FIG. 7. Lineweaver-Burk plots for [1-^{14}C] eicosa-8,11,14-trienoic acid incubation, in the absence or presence of 0.1 mM of *trans*-Δ^{10} and Δ^{13}-18:1 acids. Velocities were measured for the first 5 min of incubation at 37 C under the conditions described in the text. Each tube contained 2 mg microsomal protein of EFA-deficient rat livers.

ratios (3:1 and 6:1, respectively) used for *trans*-18:1 acids, no effect was observed (Figs. 3 and 4). These data agree with those reported by Sprecher (20) who found that 18:0 had no inhibitory effect on the desaturation of 18:2 or 20:3.

Unlabeled *trans*-18:1 acids (100 nmoles) were added to the incubation of palmitic acid. After incubation, the total lipids were separated

by thin layer chromatography in petroleum ether/diethyl ether/acetic acid (80:20:1) and the content of radioactively labeled fatty acids incorporated into each fraction was measured. The radioactivity found in the phospholipid (PL) and triglyceride (TG) fractions was decreased in the presence of *trans* acids, and this decrease was equivalent to the increase of the radioactivity in the cholesteryl ester (CE) (Table II). This phenomenon was also observed in lesser magnitude with 18:2, and was scarcely perceptible with 20:3. In these phenomena, *trans*-18:1 acids are competing with 16:0 for the same position (1-position) in microsomal PL and TG (27), but the competition is less with 18:2 and 20:3 which are predominantly incorporated at the 2-position of PL and TG. Because the total radioactivity incorporated into acyl lipids (CE + PL + TG) is the same in the absence or presence of *trans*-18:1 acids (Table II), the esterification of the substrate and its products into the microsomal lipids is not significantly affected, suggesting that the competition probably operates at the desaturation step. Stearate did not inhibit Δ5-or Δ6-desaturases, although it was incorporated into lipids into the same position as are *trans* acids. This indicates that the inhibitory effects of *trans* acids are not likely to be due to the incorporation step in the overall process, and by elimination must be expressed at the desaturation step.

Nutritional experiments indicate that *trans* fatty acids in margarine stock intensify EFA deficiency (13). Unpublished data from this laboratory indicate that the Δ6-desaturase enzyme activity is significantly decreased and the *trans* acid contents were significantly increased in liver microsomes of the rats fed *trans* acids, whereas no significant decrease in the enzyme activity was observed in rats fed an equal level of saturated fat. That study also indicated that desaturation of linoleic acid is diminished in vivo by dietary *trans* acids. Other in vivo studies showed that *trans*-9-18:1 impaired conversion of linoleic to arachidonic acid and oleic to eicosatrienoic acid (28). Results from the in vitro studies presented here offer partial explanation of that phenomenon because *trans* acids were found to decrease the Δ6-desaturase activity.

Recent studies on desaturation of *trans*-18:1 isomers revealed that they form new and unusual dienoic acids (26). These polyunsaturated fatty acids (PUFA) may be potentially competitive in the matabolism of essential fatty acids. Thus, the *trans* 18:1 acids in partially hydrogenated fats affect PUFA composition and metabolism in at least two ways.

ACKNOWLEDGMENTS

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TABLE II

The Distribution of Radioactive Fatty Acids between Acyl Lipids and the Free Fatty Acids in Microsomal Lipids[a]

	% Radioactivity in[b]				
Trans-18:1 inhibitor	PL	TG	CE	Total (PL + TG + CE)	FFA
Δ^9-Desaturase					
none	44.6	14.2	10.4	69.2	25.8
Δ^9	34.4	11.5	23.2	69.1	26.6
Δ^{12}	33.5	12.6	23.5	69.6	26.3
Δ^6-Desaturase					
none	51	21.1	3.9	76.0	20
Δ^{12}	45	17.2	9.6	71.8	26
Δ^6	47.8	19.4	8.0	75.2	23
Δ^5-Desaturase					
none	38.8	24.6	3.8	67.2	30.4
Δ^{10}	35.3	27.8	1.8	64.9	33.1
Δ^{13}	34.4	26.0	3.4	63.8	35.8

[a]The incubation mixture contained 100 nmoles of 16:0, 18:2 or 20:3 as substrates in absence or presence of 100 nmoles of *trans*-18:1 acid as inhibitor.

[b]PL, phospholipids; TG, triglycerides; CE, cholesteryl esters; FFA, free fatty acids. Each number represents an average of triplicate assays.

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Experimental Nephrotic Syndrome Induced in the Rat by Puromycin Aminonucleoside: Hepatic Synthesis of Neutral Lipids and Phospholipids from ^{3}H-Water and ^{3}H-Palmitate

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ABSTRACT

Experimental nephrotic syndrome (ascites, proteinuria, hypoalbuminemia, and hyperlipidemia) was induced in male Wistar rats by seven daily subcutaneous injections of puromycin aminonucleoside (20 mg/kg). Hepatic lipogenesis from ^{3}H-water and ^{3}H-palmitate was investigated in nephrotic and pair fed control rats by using liver slices. Total incorporation of ^{3}H-water into neutral lipids was higher in nephrotic than in control rats (413 ± 124 vs. 229 ± 46 nmoles/g/hr, $p < .01$). Among neutral lipids, the major increase was observed for triacylglycerols (106 ± 26 vs. 72 ± 21 nmoles/g/hr, $p < .05$), cholesteryl esters (3.7 ± 2.1 vs. 1.4 ± .7 nmoles/g/hr, $p < .05$) and, above all, for cholesterol (123 ± 48 vs. 36 ± 18 nmoles/g/hr, $p < .0025$). Total incorporation of ^{3}H-water into phospholipids as well as incorporation of ^{3}H-water into individual phospholipids were not significantly increased. Incorporation of ^{3}H-palmitate into neutral lipids was increased (312 ± 84 vs. 221 ± 28 nmoles/g/hr, $p < .05$). Among neutral lipids, a significant increase was observed for 1,3-diacylglycerols (19 ± 3 vs. 13 ± 3 nmoles/g/hr, $p < .025$), triacylglycerols (228 ± 50 vs. 163 ± 14 nmoles/g/hr, $p < .05$) and cholesteryl esters (18 ± 5 vs. 10 ± 1 nmoles/g/hr, $p < .01$). Incorporation of ^{3}H-palmitate into phospholipids was not significantly affected. The difference in hepatic lipogenesis between nephrotic and control rats was even more pronounced if the data were corrected for the total liver weight which was significantly increased in the nephrotic rats (11.3 ± .3 vs. 8.5 ± .1 g, $p < .001$). These findings indicate that the synthesis of neutral lipids from both ^{3}H-water and ^{3}H-palmitate is elevated in rat with aminonucleoside-induced nephrotic syndrome. The possible role of the increased hepatic lipogenesis in the pathogenesis of the nephortic hyperlipidemia is discussed.

INTRODUCTION

Although it has been known for a long time that nephrotic syndrome in man and experimental animals is associated with disturbances of the concentration and metabolism of plasma lipids and lipoproteins (1-8), the pathogenesis of nephrotic hyperlipidemia is still poorly understood.

The nephrotic syndrome induced in the rats by either the administration of antikidney serum (9) or a derivative of puromycin (10) has been used extensively to elucidate as to whether nephrotic hyperlipidemia was attributable to an increased lipid synthesis by the liver. In spite of extensive work, however, conflicting data have been reported since hepatic lipogenesis in nephrotic rats was found increased (11-16), normal (17) or even decreased (18-20).

The lack of conclusive evidence about the role of hepatic lipogenesis in the production of nephrotic hyperlipidemia prompted us to investigate this problem in rats made nephrotics by the administration of puromycin aminonucleoside. This experimental model has been adopted since the changes of plasma and urinary lipoproteins occurring in this condition have been recently characterized in our laboratory (21).

In this report we present our results obtained by investigating hepatic synthesis of neutral lipids and phospholipids from both ^{3}H-water and ^{3}H-palmitate. Specific aims of our study were: (a) to investigate the ex-novo synthesis of lipids by the liver, and (b) to measure the extent of the utilization of preformed fatty acids in the synthesis of neutral lipids and phospholipids by the nephrotic liver.

MATERIALS AND METHODS

Materials

Puromycin aminonucleoside (6-dimethylamino-9(3′-amino-3′-deoxyribosyl)purine), fatty acid poor bovine serum albumin, and standards for lipid chromatography were purchased from Sigma Chemical Co. (St. Louis, MO). Eagle's basal medium was obtained from Wellcome Research Laboratories (Beckenham, England). ^{3}H-water, ^{3}H-palmitate and scintillators (2,5-diphenyloxazole, PPO; and p-bis-(o-methylstyryl)-benzene, bis-MSB) were purchased from New England Nuclear Co. (Dreieich, West Germany). Precoated silica gel plates for thin layer chromatography were obtained from Merck (Darmstadt, West Germany). Other reagents and organic solvents were purchased from C. Erba (Milan, Italy).

Animals

Ten male Wistar rats weighing 280-310 g

were used. Before and during the experiment, rats were individually caged and kept under strictly controlled dark-light cycle. Five rats were made nephrotics by seven daily subcutaneous injections of puromycin aminonucleoside (20 mg/kg) as previously described (21). Five control rats received the same volume of distilled water. Since treatment induced a significant drop in food consumption (21), food intake was restricted in control rats on the basis of the mean daily food consumption of the nephrotic rats.

Preparation and Incubation of Liver Slices

Five days after the withdrawal of the chemical, nonfasting rats were decapitated at the middle of the dark phase and livers were rapidly removed and chilled in buffered NaCl 154 M. Liver slices (139.5 ± 21.4 mg) were prepared in duplicate from each animal and incubated in 2 ml of Eagle's basal medium containing bovine serum albumin and palmitate (.5 mM respectively). Both unlabeled and labeled palmitate were complexed to fatty acid-poor albumin according to the method of Tinker and Hanahan (22). The amount of fatty acid bound to albumin after the incubation was ca. 98% of the original amount. In the experiments in which ex novo lipid synthesis was investigated, 4 mCi of ^{3}H-water were added to each flask; in the experiments in which fatty acid utilization by liver slices was studied, 2 μCi of ^{3}H-palmitate bound to albumin were added to each flask. The incubations were carried out under an atmosphere of 95% O_2 and 5% CO_2 at 37 C in a metabolic shaker at 125 oscillations per min for 2 hr. At the end of the incubation, the content of the flasks (liver slices and medium) were transferred into an ice bath and homogenized by using a Potter Elvehjem tissue homogenizer. Homogenized material was stored at -30 C until the lipid extraction was performed (usually 1-2 weeks).

Extraction and Purification of Lipids

Hepatic Lipids were extracted and purified according to Folch et al. (23) with slight modifications. One volume of tissue homogenate was extracted with 17 volumes of chloroform/methanol (2:1, v/v) containing 2,6-di-tert-butyl-p-cresol (50 mg/ml) as antioxidant. The extract was filtered on filter paper in a separatory funnel and shaken with .2 volume of water. The lower phase was then washed 3-4 times against .2 volume of theoretical upper phase (chloroform/methanol/water, 3:48:47, v/v/v, containing $CaCl_2$ .04% in the water portion). Purified lipid extract was then taken to dryness under N_2 at 45-50 C and lipids were resuspended in a small volume of chloroform/methanol (2:1, v/v) containing the antioxidant at the concentration indicated above. We have shown in previous experiments by using ^{14}C- or ^{3}H-labeled lipids that recovery after extraction and purification was 79.1% for oleate (range 74.9-83.6), 83.3% for cholesterol (range 78.3-86.9), and 87.4 for cholesteryl oleate (range 84.7-89.6). Data presented in this report were not corrected for lipid loss during extraction and purification procedures.

Thin Layer Chromatography of Neutral Lipids and Phospholipids

Aliquots of the purified lipid extracts were applied to .25 mm thick silica gel plates prewashed with chloroform/methanol (2:1, v/v). Separation of neutral lipids was performed by using the one dimensional, two-step procedure described by Skipski et al. (24). In our hands, typical R_f values were: .16 for monoacylglycerols, .37 for cholesterol, .41 for 1,2-diacylglycerols, .44 for 1,3-diacylglycerols, .50 for fatty acids, .63 for triacylglycerols, and .73 for cholesteryl esters. Separation of phospholipids was achieved by a one dimensional procedure by using chloroform/methanol/acetic acid/water, 60:30:7:3 as developing solvent. In our conditions, we obtained the following R_f: .04 for lysophosphatidylcholine, .09 for sphingomyelin, .19 for phosphatidylcholine, .33 for phosphatidylinositol, .42 for phosphatidylserine, and .62 for phosphatidylethanolamine.

Radioactivity Measurement by Liquid Scintillation Spectrometry

After brief exposure of thin layer chromatography (TLC) plates to iodine vapors, spots containing neutral lipids and phospholipids were scraped off and transferred into scintillation vials. After the addition of a toluene-based scintillation mixture (4.9 g of PPO, .1 g of bis-MSB to 1 liter with toluene), the vials were shaken for 1 min and counted after normal light and temperature adaptation (usually 20-24 hr) in a Packard Tricarb liquid scintillation spectrometer. Counting efficiency was calculated by the automatoc external standard procedure.

Statistical Analysis

Data presented in this paper was always the mean ± standard deviation of values obtained from single animals within each experimental group. Significance was calculated by using the Student's t test.

RESULTS

Incorporation of ^{3}H-water into Hepatic Neutral Lipids and Phospholipids

The total incorporation of ^{3}H-water into hepatic neutral lipids was higher in nephrotic than in control rats (413 ± 124 vs. 229 ± 48 nmoles/g/hr, $p < .01$). As shown in Figure 1, we found an increased incorporation of ^{3}H-water into triacylglycerols ($p < .05$), cholesteryl esters ($p < .05$), and above all into cholesterol ($p < .0025$) by the liver slices of nephrotic rats. When we expressed the radioactivity of each lipid class as percentage of the total ^{3}H-water incorporated into lipids, we found a marked elevation of the radioactivity incorporated into cholesterol by nephrotic liver (Table I). The total incorporation of ^{3}H-water into phospholipids was not significantly increased in nephrotic rats (113 ± 20 vs. 103 ± 19 nmoles/g/hr). The radioactivity of each phospholipid class measured as percentage of the total ^{3}H-water incorporated into phospholipids was approximately the same in nephrotic and control rats.

Incorporation of ^{3}H-palmitate into Hepatic Neutral Lipids and Phospholipids

The incorporation of ^{3}H-palmitate into neutral lipids was increased in nephrotic rats (312 ± 84 vs. 221 ± 28 nmoles/g/hr, $p < .05$). As shown in Figure 2, the major increase was observed ror 1,3-diacylglycerols ($p < 0.5$), triacylglycerols ($p < .05$), and cholesteryl esters ($p < .01$). The synthesis of total phospholipids from ^{3}H-palmitate was not significantly increased in nephrotic rats (38 ± 8 vs. 33 ± 8 nmoles/g/hr), nor was the incorporation of the precursor into the various phospholipid classes.

DISCUSSION

In this study we investigated the synthesis of neutral lipids and phospholipids by liver slices of nephrotic rats at the stage of full blown disease when edema, proteinuria, hypoalbuminemia and hyperlipidemia were present (21).

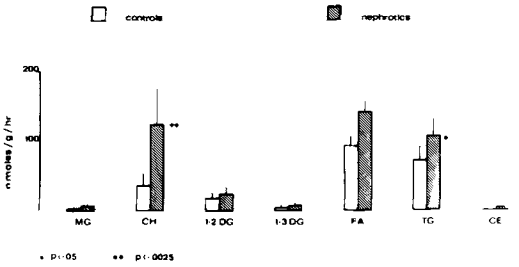

FIG. 1. Incorporation of ^{3}H-water into neutral lipids by liver slices from control and nephrotic rats. Data are mean ± standard deviation of values obtained from single animals within each experimental group.

Hepatic lipogenesis was studied by incubating liver slices with two different lipid precursors, namely ^{3}H-water and ^{3}H-palmitate. By this approach, one can investigate both the ex novo lipogenesis (i.e., the incorporation of ^{3}H-water) and the lipogenesis which results from the utilization of preformed fatty acids (incorporation of ^{3}H-palmitate). The reason for exploring both pathways is based on the following considerations: a) nephrotic syndrome may be associated with an increased activity and/or concentration of several key enzymes of both metabolic pathways, and b) since the concentration of plasma fatty acids is increased in nephrotic syndrome (21), and it is well recognized that the amount of fatty acids reaching the liver represents a stimulus for the hepatic synthesis of triacylglycerol rich plasma lipoproteins (25-27), one could expect that in nephrotic liver a greater proportion of preformed fatty acids is channelled into lipids.

As far as the methodology employed in this study is concerned, three points should be emphasized: (a) the incorporation of both ^{3}H-water and ^{3}H-palmitate was carried out by incubating liver slices in a medium containing

TABLE I

Percentage Incorporation of ^{3}H-water into Hepatic Neutral Lipids

	MG	CH	1,2-DG	1,3-DG	FA	TG	CE
Controls	1.6 ± 0.8	15.2 ± 6.1	8.0 ± 0.4	1.7 ± 1.2	41.4 ± 7.2	31.5 ± 6.0	0.6 ± 0.2
Nephrotics	1.4 ± 0.4	29.6 ± 7.0[a]	5.8 ± 1.3	1.7 ± 0.7	34.3 ± 9.9	26.3 ± 3.8	0.9 ± 0.6

NOTE: The figures represent the percentage of total ^{3}H-water incorporated into neutral lipids found in each lipid class.

[a] $p < .005$.

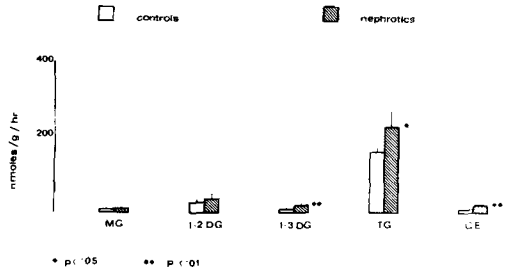

FIG. 2. Incorporation of ^{3}H-palmitate into neutral lipids by liver slices from control and nephrotic rats. Data are mean ± standard deviation of values obtained from single animals within each experimental group.

the same concentration of fatty acids which approximated that found in normal rat plasma; (b) ex novo lipogenesis was studied by using ^{3}H-water instead of ^{14}C-acetate in view of the limitations involved in the use of the latter precursor (28-29); and (c) in the study of the incorporation of ^{3}H-palmitate we assumed that the dilution of the label in the hepatic pool of palmitate was the same in both control and nephrotic rats. Our results clearly indicate that the hepatic synthesis of neutral lipids from both ^{3}H-water and ^{3}H-palmitate was increased in nephrotic rats. The synthesis of phospholipids showed only a minor increase which was not statistically significant. The difference observed between control and nephrotic rats is even more pronounced if one takes into account that nephrotic liver is heavier than control liver (11.3 ± .3 vs. 8.5 ± .1 g, $p < 0.001$). If hepatic lipogenesis is corrected for the liver weight, the synthesis of neutral lipids from ^{3}H-water and ^{3}H-palmitate in nephrotic rats would be 2.4-and 1.9-fold, respectively, the value found in the control rats. On the other hand, the synthesis of phospholipids from both precursors would be 1.45- and 1.4-fold, respectively.

The increased lipogenesis found in nephrotic liver may be due to several factors. It is likely that the activity of some rate-limiting enzymes is increased. One may postulate, for example, that the incorporation of ^{3}H-water into cholesterol may result from an increase of microsomal HMG-CoA reductase (EC 1.1.1.34) since several reports indicate that changes in the incorporation of ^{3}H-water into cholesterol by liver tissue are paralleled by concomitant and similar changes of the activity of HMG-CoA reductase (30).

The increased utilization of preformed fatty acids by nephrotic liver for the synthesis of lipids in our in vitro systems where the concentration of fatty acids in the medium was kept constant and within physiological levels, may be related to the following factors: (a) an increased uptake of fatty acids through the liver plasma membrane or, most likely, an increased intracellular transport of these compounds due to an elevation of the fatty acid binding protein (31-32); and (b) an increased activity of the enzymes involved in the synthesis of long chain fatty acid acyl-CoA thioesters or in the transfer of the fatty acyl-CoA to glycerol and cholesterol.

In most of the previous studies, hepatic lipogenesis of nephrotic rats was found to be either elevated (11-16) or depressed (18-20). Although the reasons for this discrepancy are not clearly understood, the following points should be taken into account: (a) since, during the development of the disease in the rat there is a drop in the consumption of food (21), hepatic lipid synthesis in nephrotic animals should be compared to that found in livers of pair-fed rats in view of the effect of the low calorie intake on lipid metabolism; unfortunately, it seems to us that since in most of the previous studies no pair-fed rats were used as controls, (b) the stage of the disease at which the study on lipogenesis is carried out may be crucially important. Studies carried out by the same group (11,18) indicate that, in the early stages of the nephrotic syndrome induced by antikidney serum, hepatic lipogenesis was increased (11), whereas the opposite was true at the later stage of the disease (18).

It is of interest to compare the data on hepatic lipogenesis found in nephrotic rats with the changes of plasma lipids and lipoproteins we described in rats which were subjected to the same experimental protocol. In these animals we found a marked increased of plasma cholesterol, cholesteryl esters and phospholipids (5- to 6-fold) and triacylglycerols (7- to 8-fold (21). If one compares the data shown in Figures 1 and 2 with those of plasma lipids, it clearly emerges that the increased hepatic lipogenesis, as measured in vitro, can only in part account for the elevated plasma lipid concentration. It should be pointed out, however, that the lipogenesis of nephrotic liver in vivo may be higher than expected on the basis of the study in vitro. In the in vivo situation, nephrotic liver is perfused with plasma which contains a level of fatty acids which is about 2-2.5 times greater than that found in plasma of pair-fed controls. It may be possible that the high fatty acid concentration in nephrotic plasma may further stimulate hepatic lipogenesis in vivo. It cannot be ruled out, however, that nephrotic plasma contains other

factors which are capable of stimulating hepatic lipogenesis.

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Biosynthesis of Cholic Acid in Rat Liver: Formation of Cholic Acid from 3α, 7α, 12α-Trihydroxy- and 3α, 7α, 12α, 24-Tetrahydroxy-5β-Cholestanoic Acids

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ABSTRACT

Conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into 3α,7α,12α24-tetrahydroxy-5β-cholestanoic and cholic acids was catalyzed either by the mitochondrial fraction fortified with coenzyme A, ATP, $MgCl_2$ and NAD or by the combination of microsomal fraction and 100,000 x g supernatant fluid fortified with coenzyme A, ATP and NAD. 24-Hydroxylation and formation of cholic acid occurred at similar rates with the 25R- and the 25S-forms of 3α,7α,12α-trihydroxy-5β-cholestanoic acid. The 25R- and 25S-forms of 3α,7α,12α-trihydroxy- and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acids were administered to bile fistula rats. Labeled cholic acid was isolated from the bile. The initial specific radioactivity of cholic acid was higher and the disappearance of radioactivity more rapid after administration of 3α,7α,12α-trihydroxy-5β-cholestanoic acid than of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. The findings are discussed in relation to the assumed pathway for side chain cleavage in cholic acid biosynthesis.

INTRODUCTION

According to current concepts, the nuclear hydroxylations precede side chain degradation in the biosynthesis of cholic acid in rat liver. 5β-Cholestane-3α,7α,12α-triol is generally regarded as the major substrate for 26-hydroxylation, the initial side chain hydroxylation (1). In the next steps, 5β-cholestane-3α,7α,12α,26-tetrol is oxidized to 3α,7α,12α-trihydroxy-5β-cholestanoic acid (1). The further conversion into cholic acid probably involves a 24-hydroxylation yielding 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (2).

Bergström et al. (3) and Briggs et al. (4) showed that the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid occurs in rat liver homogenate and rat liver mitochondria, respectively. Recently, Hanson et al. (5) have shown the same reaction in homogenates of human livers. Early studies by Masui and Staple (6) showed that 24-hydroxylation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid can be catalyzed by the mitochondrial fraction in combination with the 100,000 x g supernatant fluid. Recent experiments in this laboratory have revealed a more efficient 24-hydroxylating activity towards 3α,7α,12α-trihydroxy-5β-cholestanoic acid by a combination of microsomal fraction and 100,000 x g supernatant fluid (2). The activity was shown to be due to the combined effect of a desaturase and a hydratase and was dependent upon ATP (2).

The conversion of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid into cholic acid has been shown to occur in vivo in the guinea pig and in vitro in mitochondrial and soluble preparations from rat liver homogenate (7,8). The aim of the present investigation was to study the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid in more detail, especially with regard to subcellular localization, cofactor requirements and the possible role of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid as an intermediate.

EXPERIMENTAL PROCEDURE

Labeled Compounds

3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]cholestanoic acid (specific radioactivity, 0.2 mCi/μmol) was prepared by reduction with tritium-labeled sodium borohydride (Radiochemical Centre, Amersham, England) of 3α,12α-dihydroxy-7-oxo-5β-cholestanoic acid, prepared from 3α,7α,12α-trihydroxy-5β-cholestanoic acid by oxidation with N-bromosuccinimide (2). The purity of the material as checked by thin layer chromatography and radio gas chromatography was more than 99.9%. The compound consisted predominantly of the 25S-form (see unlabeled compounds).

25R-3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]cholestanoic acid (specific radioactivity, 1.3 mCi/μmol) and 25S-3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid (specific radioactivity, 1.3 mCi/μmol) were prepared from the corresponding unlabeled compounds as described above (2).

3α,7α,12α,24-Tetrahydroxy-5β-[7β-^{3}H] cholestanoic acid (specific radioactivity, 0.2 mCi/μmol) was prepared biosynthetically from 3α,7α,12α-trihydroxy-5β-[7β-^{3}H] cholestanoic acid of the same specific radioactivity (see above) by incubation with microsomal and soluble fractions of rat liver homogenate fortified with ATP. The material was purified by thin layer chromatography and the identity checked by gas chromatography-mass spectrometry (c.f. ref. 2). The radioactive purity of the material was more than 99.9%. Biosynthetically prepared 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid consists of one of the C-24 epimers, probably the 24α-epimer (2). 25R-3α,7α,12α,24-Tetrahydroxy-5β-[7β-^{3}H] cholestanoic acid (specific radioactivity, 1.3 mCi/μmol) and 25S-3α,7α,12α,24-tetrahydroxy-5β-[7β-^{3}H] cholestanoic acid (specific radioactivity, 1.3 mCi/μmol) were prepared biosynthetically from the corresponding 3α,7α,12α-trihydroxy acids as above. The purity was more than 99.9%.

Unlabeled Compounds

3α,7α,12α-Trihydroxy-5β-cholestanoic acid was isolated from a sample of bile of a Caiman species as described earlier (2). The melting point (187-188 C) suggested that the compound consisted predominantly of the 25S-form (2). 25R- and 25S-3α,7α,12α-Trihydroxy-5β-cholestanoic acids were generous gifts from Dr. Thomas Briggs (9). 3α,7α,12α,24-Tetrahydroxy-5β-cholestanoic acid was synthesized according to Inai et al. (10). The identity of the material was confirmed by gas chromatography-mass spectrometry. 3α,7α,12α,24-Tetrahydroxy-5β-cholestanoic acid prepared in this way consists of equal parts of the two C-24-epimers as well as the 25R- and 25S-forms. The C-24-epimer corresponding to the biosynthesized compound (probably 24α) was isolated from the synthesis mixture by thin layer chromatography (c.f. 2).

Cofactors

NAD, ATP, AMP and coenzyme A were obtained from Sigma Chemical Co. (St. Louis, MO).

METHODS

Enzyme Fractions

Male rats of the Sprague-Dawley strain weighing ca. 200 g were used. Liver homogenates, 20% (w/v), were prepared in 0.1 M Tris-Cl buffer pH 8.5 for the preparation of the microsomal fraction (6 mg of protein/ml) and 100,000 x g supernatant fraction (20 mg of protein/ml) (11).

The mitochondrial fraction was prepared according to Sottocasa et al. (12). The final suspension was made in 0.1 M Tris-Cl buffer, pH 8.5. A mitochondrial fraction prepared in this way had a protein concentration of ca. 5 mg/ml. Protein determinations were performed according to Lowry et al. (13).

Incubation Procedure and Analysis of Incubation Mixtures

In a standard incubation procedure, 3α,7α,12α-trihydroxy-5β-[7β-^{3}H] cholestanoic acid, 50 μg dissolved in 25 μl of methanol, was incubated for 40 min at 37 C with 0.5 ml of subcellular preparation in a total volume of 3 ml of 0.1 M Tris-Cl buffer, pH 8.5. The incubation mixture was fortified with NAD, 3 μmol, ATP, 7 μmol and coenzyme A, 2.6 μmol. $MgCl_2$, 30 μmol, was added in incubations with the mitochondrial fraction. All incubations were terminated by the addition of 5 ml of 95% (v/v) aqueous ethanol. The reaction mixture was diluted with water, acidified and extracted twice with ethyl acetate. The combined ethyl acetate extracts were washed with water until neutral. In some cases, the reaction mixtures were hydrolyzed with 1 M NaOH in 50% (v/v) aqueous ethanol at 110 C for 10 hr. The residues of the ethyl acetate extracts were subjected to thin layer chromatography using solvent system S7 (14). The extent of conversion was calculated from measurements of the peak area of the radioactivity tracings obtained by scanning with a thin layer scanner (Berthold, Karlsruhe, Germany). In some cases, the chromatographic zone corresponding to cholic acid was eluted with methanol and crystallized to constant specific radioactivity after addition of unlabeled cholic acid.

In Vivo Experiments

Male rats of the Sprague-Dawley strain weighing ca. 200 g were used for bile duct cannulation according to Fisher and Vars (15). Before administration of the first compounds, the bile was diverted and discarded for 3 days. The labeled compounds were administered intraperitoneally in an emulsion of a 1% (w/v) solution of serum albumin in 10% (v/v) aqueous ethanol. The bile samples were hydrolyzed in 2 M NaOH at 110 C for 10 hr. The hydrolysates were acidified and extracted with ethyl acetate. The residues of the ethyl acetate extracts were subjected to reversed phase partition chromatography using phase system F1 (16). The material in the trihydroxy fraction was rechromatographed in phase system C1 (16). A steady state with regard to bile acid production during

the experimental period was ascertained by the finding of a constant bile acid secretion rate as judged by data from the reversed phase partition chromatographies.

RESULTS

Conversion of 3α,7α,12α-Trihydroxy-5β-Cholestanoic Acid into 3α,7α,12α,24-Tetrahydroxy-5β-Cholestanoic Acid and Cholic Acid

3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]cholestanoic acid was incubated with different subcellular fractions of rat liver homogenate. With appropriate cofactors, the formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid was found in 800 x g supernatant, mitochondrial fraction and the combination of microsomal fraction and 100,000 x g supernatant fluid. In some experiments, no or very little 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was detected. Table I shows the enzymatic activity of different subcellular fractions as well as cofactor requirements. With the mitochondrial fraction, formation of both products was obtained with the addition of ATP, NAD, $MgCl_2$ and coenzyme A. Omission of NAD increased the amount of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid obtained, whereas cholic acid formation decreased.

Omission of ATP or coenzyme A decreased both the formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and of cholic acid. Omission of Mg^{2+} decreased the formation of both acids by 50%. In earlier work concerning metabolism of 3α,7α,12α-trihydroxy-5β-cholestanoic acid, glutathione, AMP and citrate were added to the incubation mixtures (4). In the present work, none of these cofactors was found to influence the mitochondrial conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid. Addition of microsomal fraction to the mitochondrial incubations did not change the conversions whereas a marked inhibition in cholic acid formation was noted when 100,000 x g supernatant fluid was added. In agreement with earlier findings, the amount of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid increased when 100,000 x g supernatant fluid was added (2).

When the microsomal fraction or the 100,000 x g supernatant fluid was incubated alone, no formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid or cholic acid was detected. Combination of the microsomal fraction and 100,000 x g supernatant fluid fortified with ATP, coenzyme A and NAD yielded both 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid. When ATP was omitted no 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was detected, whereas formation of cholic acid was unaffected or somewhat decreased. NAD was necessary for cholic acid formation, whereas omission of coenzyme A increased 24-hydroxylation as well as cholic acid formation. Saponification of the incubation mixtures did not increase the yields of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid or cholic acid neither in incubations with the mitochondrial fraction nor in those with the combined microsomal and cytoplasmic fractions.

Tables II and III show the results of crystallizations to constant specific radioactivity of labeled material obtained from thin layer chromatograms of incubations of 3α,7α,12α-trihydroxy-5β-cholestanoic acid with the mitochondrial fraction supplemented with cofactors and the combination of microsomal fraction and 100,000 x g supernatant fluid fortified with cofactors. The results in Tables II and III establish the identity of the labeled material with cholic acid. The identity and radioactive purity of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was ascertained by thin layer chromatography, radio gas chromatography and gas chromatography - mass spectrometry as described earlier (2).

*Conversion of 25R- and 25S-*3α,7α,12α-Trihydroxy-5β-Cholestanoic Acid into 3α,7α,12α,24-Tetrahydroxy-5β-Cholestanoic and Cholic Acids

Table IV shows that the mitochondrial fraction converts the two C-25 stereoisomers of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic and cholic acids at similar rates. The relative conversions were lower in experiments with the combination of microsomal fraction and 100,000 x g supernatant fluid, but the two C-25 isomers were metabolized at the same rate.

Effect of Time, Enzyme and Substrate Concentration on Conversion of 3α,7α,12α-Trihydroxy-5β-Cholestanoic Acid into 3α,7α,12α,24-Tetrahydroxy-5β-Cholestanoic Acid and Cholic Acid

Mitochondrial formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid was linear with time for at least 40 min (Fig. 1A). The rate of reaction increased linearly with increasing amounts of mitochondrial protein up to ca. 5 mg (Fig. 1B). Under standard conditions, ca. 50 μg saturated the system both with regard to the formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid

and cholic acid (Fig. 1C). In some series of incubations, no 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid could be detected. In such cases, the water phase was analyzed for radioactivity. No significant amounts of radioactivity were found in this phase indicating that the absence of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was not due to an incomplete extraction. Figure 2A shows that the formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid in the combination of

TABLE I

Conversion of 3α,7α,12α-Trihydroxy-5β-Cholestanoic Acid into 3α,7α,12α,24-Tetrahydroxy-5β-Cholestanoic Acid and Cholic Acid by Subcellular Fractions with Different Cofactor Additions[a]

	Conversion pmol/min	
	3α,7α,12α,24-Tetrahydroxy-5β-cholestanoic acid	Cholic acid
Mitochondrial fraction	20	191
Mitochondrial fraction ATP excluded	<14	20
Mitochondrial fraction NAD excluded	35	22
Mitochondrial fraction $MgCl_2$ excluded	<14	78
Mitochondrial fraction coenzyme A excluded	<14	50
Mitochondrial fraction + Microsomal fraction	20	176
Mitochondrial fraction + Microsomal fraction Coenzyme A excluded	<14	59
Mitochondrial fraction + 100,000 x g supernatant fluid	31	<14
Mitochondrial fraction + 100,000 x g supernatant fluid Coenzyme A excluded	<14	31
Microsomal fraction + 100,000 x g supernatant fluid	20	53
Microsomal fraction + 100,000 x g supernatant fluid Coenzyme A excluded	80	60
Microsomal fraction + 100,000 x g supernatant fluid NAD excluded	20	<14
Microsomal fraction + 100,000 x g supernatant fluid ATP excluded	<14	54
Microsomal fraction + 100,000 x g supernatant fluid $MgCl_2$ added	<14	31
Microsomal fraction	<14	<14
Microsomal fraction Coenzyme A excluded	<14	<14
100,000 x g supernatant fluid	<14	<14
100,000 x g supernatant fluid Coenzyme A excluded	<14	<14

[a]The amounts of protein added were: mitochondrial fraction, 2.5 mg, microsomal fraction, 3.0 mg, and 100,000 x g supernatant fluid, 10 mg.

TABLE II

Identification of Cholic Acid from Incubations with the Mitochondrial Fraction[a]

Solvent	Number of crystallization	Weight mg	Specific radioactivity Counts x 10^3/min^{-1} mg^{-1}
None	0	25.9	40.8
Methanol-water	1	20.7	40.5
Methanol-water	2	16.6	42.2
Methanol-water	3	13.2	45.3

[a]Cholic acid was isolated by means of thin layer chromatography from extracts of incubations of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid with the mitochondrial fraction and cofactors as described under "Methods."

TABLE III

Identification of Cholic Acid from Incubations with the Microsomal Fraction and 100,000 x g Supernatant Fluid[a]

Solvent	Number of crystallization	Weight mg	Specific radioactivity Counts x 10^3/min^{-1} mg^{-1}
None	0	24.4	21.1
Methanol-water	1	17.7	24.5
Methanol-water	2	11.6	24.6
Methanol-water	3	8.4	27.7

[a]Cholic acid was isolated by means of thin layer chromatography from extracts of incubations of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic aicd with the microsomal fraction and 100,000 x g supernatant fluid fortified with cofactors as described under "Methods."

TABLE IV

Conversion of 25R- and 25S-3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]Cholestanoic Acid into 3α,7α,12α,24-Tetrahydroxy-5β-Cholestanoic Acid and Cholic Acid in Subcellular Fractions of Rat Liver[a]

	Conversion %	
	3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid	Cholic acid
Mitochondrial fraction, 25S-3α,7α,12α-trihydroxy-5β-cholestanoic acid	2.5	67.3
Mitochondrial fraction, 25R-3α,7α,12α-trihydroxy-5β-cholestanoic acid	1.7	43.0
Microsomal fraction 100,000 x g supernatant fluid, 25S-3α,7α,12α-trihydroxy-5β-cholestanoic acid	0.8	10.0
Microsomal fraction, 100,000 x g supernatnat fluid, 25R-3α,7α,12α-trihydroxy-5β-cholestanoic acid	1.2	8.9

[a]3α,7α,12α-Trihydroxy-5β-cholestanoic acid, 5 μg, was added to each incubation.

microsomal fraction and 100,000 x g supernatant fluid is linear with time for at least 40 min. The rate of reaction increased linearily with increasing amounts of protein up to 13 mg (Fig. 2B) (corresponding to 0.5 ml of microsomal fraction and 0.5 ml of 100,000 x g supernatant fluid). Fifty μg of 3α,7α,12α-trihydroxy-5β-cholestanoic acid saturated the system with respect to the formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. Addition of substrate up to 130 μg did not saturate the system with respect to formation of cholic acid (Fig. 2C).

In separate experiments, the effect of addition of increasing amounts of unlabeled 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid to incubations of 3α,7α,12α-trihydroxy-5β-cholestanoic acid with the mitochondrial or microsomal and 100,000 x g supernatant fractions was tested. Addition of unlabeled 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid up to 100 μg lowered the amount of cholic acid obtained in a linear way. Addition of 100 μg of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid lowered the formation of cholic acid by ca. 40% in mitochondrial incubations. The corresponding value for the combined microsomal and 100,000 x g supernatant fractions

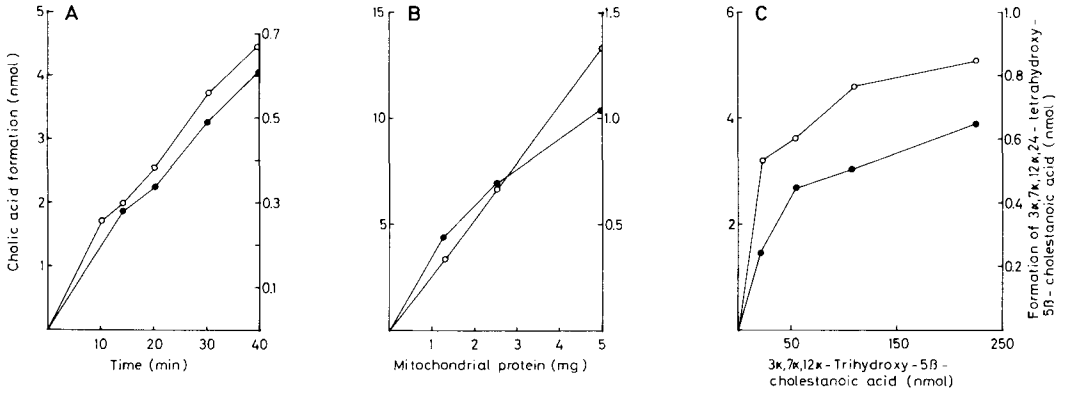

FIG. 1. Effect of time (A), mitochondrial protein concentration (B) and substrate concentration (C) on formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid from 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid. Incubations were performed for 40 min except in (A); 2.5 mg of mitochondrial protein was used except in (B); 50 μg of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid were added except in (C). ○, cholic acid; ●, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid.

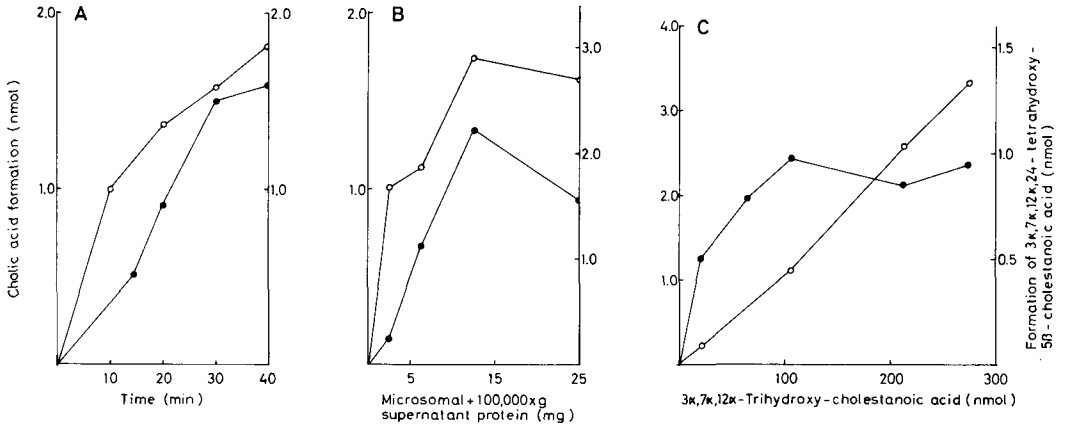

FIG. 2. Effect of time (A), microsomal-100,000 x g supernatant protein (B) and substrate concentration (C) on formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid from 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid. Incubations were performed for 40 min except in (A), 13 mg of mitochondrial-100,000 x g supernatant protein (corresponding to 0.5 ml of each subcellular fraction) were used except in (B), 50 μg of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid were added except in (C). ○, cholic acid; ●, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid.

was 30%.

Conversion of 3α,7α,12α,24-Tetrahydroxy-5β-Cholestanoic Acid into Cholic Acid in vitro

3α,7α,12α,24-Tetrahydroxy-5β-[7β-^{3}H]cholestanoic acid was prepared biosynthetically from 3α,7α,12α-trihydroxy-5β-cholestanoic acid by incubations with the microsomal and 100,000 x g supernatant fractions in combination with ATP. In experiments concerning metabolism of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid, sometimes a rather high degree of autoxidation occurred as judged by blank incubations. Both the mitochondrial fraction and the combination of microsomal fraction and 100,000 x g supernatant fluid catalyzed conversion of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid into cholic acid (Table V). In many experiments, cholic acid formation was more efficient with 3α,7α,12α-trihydroxy-5β-cholestanoic acid as substrate than with 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. When 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was incubated with only the microsomal fraction or the 100,000 x g supernatant fluid, significant conversion into cholic acid was obtained with the microsomal fraction. The activity of the 100,000 x g supernatant fluid did not increase after dialysis removing endogenous cholic acid.

Conversion of 3α,7α,12α-Trihydroxy-5β-Cholestanoic and 3α,7α,12α,24-Tetrahydroxy-5β-Cholestanoic Acids into Cholic Acid in vivo

Figures 3 and 4 show the specific radio-

TABLE V

Conversion of 3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]-Cholestanoic Acid and 3α,7α,12α,24-Tetrahydroxy-5β-[7β-^{3}H]Cholestanoic Acid into Cholic Acid[a]

	Conversion %
Mitochondrial fraction, 3α,7α,12α-trihydroxy-5β-cholestanoic acid	41.9
Microsomal fraction, 100,000 x g supernatnat fluid, 3α,7α,12α-trihydroxy-5β-cholestanoic acid	11.7
Mitochondrial fraction, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid	7.0
Microsomal fraction, 100,000 x g supernatant fluid, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid	9.3

[a]3α,7α,12α-Trihydroxy-5β-cholestanoic acid, 3 μg, and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid, 3 μg, were used.

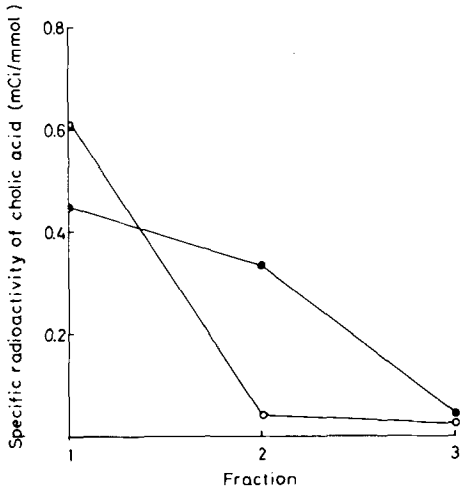

FIG. 3. Specific radioactivity of cholic acid after injection of 25R-3α,7α,12α-trihydroxy-5β-[7β-^{3}H]-cholestanoic acid and 25R-3α,7α,12α,24-tetrahydroxy-5β-[7β-^{3}H]cholestanoic acid into one bile fistula rat. The compounds had the same specific radioactivity, 1.3 mCi/μmol. The amount of radioactivity injected was 5 μCi for both compounds. 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid was injected 3 days after operation and 3α,7α,12α,24-tetrahydroxy-5β-[7β-^{3}H]-cholestanoic acid was injected into the same rat 5 days after operation. The first fraction corresponds to bile secreted during the first 3 hr after injection. The intervals between the following fractions are 3 hours. ○, cholic acid obtained after injection of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestanoic acid. ●, cholic acid obtained after injection of 3α,7α,12α,24-tetrahydroxy-5β-[7β-^{3}H]cholestanoic acid.

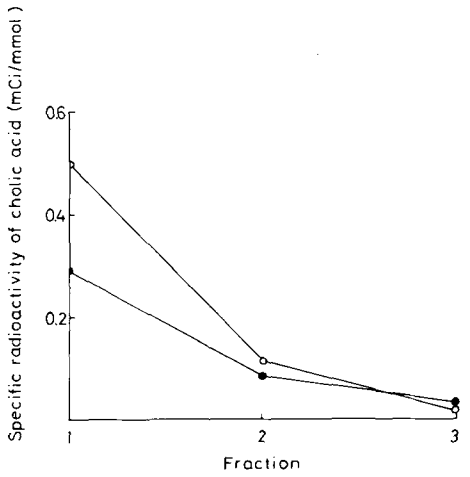

FIG. 4. Specific radioactivity of cholic acid after injection of 25S-3α,7α,12α-trihydroxy-5β-[7β-^{3}H]-cholestanoic acid and 25S-3α,7α,12α,24-tetrahydroxy-5β-[7β-^{3}H]cholestanoic acid into one bile fistula rat. The compounds had the same specific radioactivity, 1.3 mCi/μmol. The amount of radioactivity injected was 5 μCi for both compounds. For experimental details see Figure 3.

activity of cholic acid obtained from two bile fistula rats after administration of 3α,7α,12α-trihydroxy-5β-cholestanoic acid followed by an equal amount of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid 48 hr later. The administered compounds had the same specifc radioactivity. The initial specific radioactivity was somewhat higher and the disappearance of radioactivity was more rapid after administration of 25R- and 25S-3α,7α,12α-trihydroxy-5β-cholestanoic acid than of the corresponding 24-hydroxy derivatives.

DISCUSSION

The present work describes studies on the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid in rat liver with special regard to the assumed role of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid as an intermediate. The results confirm findings by Briggs et al. (4), Masui and Staple (6,8) and Gustafsson (2) concerning the ability of the mitocondrial fraction to catalyze the formation of cholic acid and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid. Considerable variation in the amount of product formed was noted, especially of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid.

The present work also reports formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid in the combination of microsomal fraction and 100,000 x g supernatant fluid. In agreement with early in vivo findings by Bridgwater and Lindstedt (17), there were no marked differences in conversion of 25R- and 25S-3α,7α,12α-trihydroxy-5β-cholestanoic acid into 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid and cholic acid either in the mitochondrial fraction or in the combined microsomal and 100,000 x g supernatant fractions.

It might be added that work in this laboratory has shown a marked similarity between the metabolism in vitro of 3α,7α-dihydroxy-5β-cholestanoic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid. The formation of the 24-hydroxy derivative and of chenodeoxycholic acid from 3α,7α-dihydroxy-5β-cholestanoic acid was catalyzed by the same subcellular fractions, and the cofactor requirements were the same (18, and Gustafsson, unpublished observations).

The present results do not provide an unequivocal answer to the question of whether or not 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid is an obligatory intermediate in the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid. The following points should be considered.

1. A precursor-intermediate-product relationship in the enzyme kinetics could not be found either in incubations with the mitochondrial fraction or in those with the combination of microsomal fraction and 100,000 x g supernatant fluid. Addition of unlabeled 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid inhibited the formation of cholic acid to a significant extent, but in several experiments no radioactive 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was found in spite of the attempt to "trap" the compound by addition of unlabeled material. Further, the omission of ATP, a cofactor known to be obligatory for 24-hydroxylation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid, did not significantly decrease cholic acid formation in the microsomal-cytoplasmic system (2). This would speak against 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid as the only obligatory intermediate in cholic acid biosynthesis in this system.

2. The direct conversion of biosynthesized 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid into cholic acid was studied in vitro. Biosynthesized 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid consists of one epimer, probably the 24α-epimer (2,6). No evidence for the formation of the 24β-epimer has been presented with in vitro preparations. Both with the mitochondrial fraction and the combination of microsomal fraction and 100,000 x g supernatant fluid, the formation of cholic acid was usually more efficient from the assumed precursor, i.e., 3α,7α,12α-trihydroxy-5β-cholestanoic acid, than from its 24-hydroxy-derivative. In contrast to the finding of Masui and Staple (8), the 100,000 x g supernatant fluid converted 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid into cholic acid poorly. Masui and Staple (8) mention the possibility that the soluble enzyme in their investigation may come from disintegrated mitochondria.

3. The in vivo formation of cholic acid from 3α,7α,12α-trihydroxy- and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid was assayed in bile fistula rats that were in a steady state regarding bile acid production. The same rat was given either 25R- or 25S-3α,7α,12α-trihydroxy-5β-cholestanoic acid followed by the corresponding 24-hydroxy acid. Both with the 25R- and 25S-acids, the initial specific radioactivity of cholic acid was somewhat higher with 3α,7α,12α-trihydroxy-5β-cholestanoic acid than with 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. The disappearance of cholic acid

radioactivity from bile was also more rapid with 3α,7α,12α-trihydroxy-5β-cholestanoic acid than with the 24-hydroxy derivative. This was particularly marked with the 25R-acids.

The three points discussed above may be interpreted to indicate the existence of an additional pathway in the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid not involving 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. On the other hand, no metabolites except 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid have been detected in in vitro preparations. Further, the differences in metabolism of 3α,7α,12α-trihydroxy-5β-cholestanoic acid and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid could be due to a difference between the tri- and tetrahydroxy acids in permeation through the membranes to the site of enzyme action.

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10α-Cucurbita-5, 24-dien-3β-ol from Gourd Seed Oil

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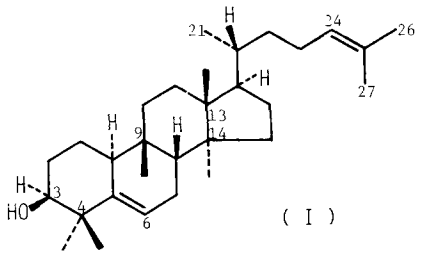
ABSTRACT

10α-Cucurbita-5,24-dien-3β-ol was isolated from the unsaponifiable matter of the seed oil of gourd (*Lagenaria leucantha* var. *Gourda;* Cucurbitaceae); it has been previously synthesized, but never found in the plant kingdom. The triterpene alcohol represents the parent compound of a number of cucurbitacins, highly oxygenated tetracyclic triterpenes found in Cucurbitaceae and some other flowering plants.

INTRODUCTION

Previously, a triterpene alcohol was isolated from the unsaponifiable matter (USM) of the seed oil of gourd (*Lagenaria siceraria;* Cucurbitaceae) by Jeong et al., but its structure remained undetermined (1). We now report here the reisolation of the triterpene alcohol from the USM of the seed oil of gourd (*L. leucantha* var. *Gourda*) and the identification of its structure as 10α-cucurbita-5,24-dien-3β-ol (anhydrolitsomentol, **I**) which has not been found as a natural component in the plant kingdom, though previously been synthesized by dehydration from litsomentol (10α-cucurbit-24-en-3β,5α-diol) (2).



(I)

EXPERIMENTAL

General

Crystallizations were performed in methanol. Melting points (mp) taken on a heat block were uncorrected. IR spectra were recorded in KBr on an IRA-2, IR spectrophotometer (Japan Spectroscopic Co., Tokyo). ^{1}H-NMR spectra were obtained with a JNM-FX 100 instrument (Japan Electron Optics Laboratory Co., Tokyo) at 100 MHz in deuteriochloroform ($CDCl_3$). The chemical shifts are given in δ with tetramethylsilane as internal standard. Lanthanide-induced shifts (LIS) of proton signals were obtained in the presence of a molar equivalent of *tris*(dipivaloylmethanato)europium (Eu[dpm]$_3$), a lanthanide shift reagent, in the similar manner as described previously (3,4). Paramagnetic induced shifts (ΔEu values) for each proton signal were then determined by the equation defined as $\delta^{Eu(dpm)_3}_{n=1} - \delta_{CDCl_3}$, where $\delta^{Eu(dpm)_3}_{n=1}$ is the LIS of proton signals at the molar ratio of Eu(dpm)$_3$ to substrate is 1, and the largest value among the observed ΔEu values for the respective methyl signals on addition of Eu(dpm)$_3$ in each compound was further normalized to a value of 100 (4). Mass spectra (MS, 70 eV) were taken on a Hitachi RMU-7M mass spectrometer with a direct inlet system (Hitachi Ltd., Tokyo). Gas liquid chromatography (GLC) for triterpene acetate was performed with a Shimadzu GC-4CM instrument (Shimadzu Seisakusho Ltd., Kyoto) equipped with a hydrogen flame ionization detector. Poly I-110 (column 270 C) and OV-17 (column 260 C) SCOT glass capillary columns (30 m x 0.3 mm I.D., Wako Pure Chemical Industries Ltd., Osaka) were used, and the relative retention time (RRT) of triterpene acetate was given relative to cholesterol acetate. Preparative thin layer (0.5 mm thick) chromatography (TLC) on silica gel of USM was developed 3 times with *n*-hexane-ethyl ether (7:3).

Gourd (*L. leucantha* var. *Gourda*) seeds were courteously supplied from Sakata Seeds Co., Yokohama, and the authentic specimen of I-acetate (10α-cucurbita-5,24-dien-3β-ol acetate; anhydroacetyllitsomentol) was generously donated by Dr. N. Viswanathan, Ciba-Geigy Research Center, Bombay (India).

Isolation of a Triterpene Alcohol (I) from Gourd Seed Oil

Saponification of gourd seed oil (376 g), extracted from dried and ground seeds (1900 g) with methylene chloride using a Soxhlet extractor, afforded USM (4.78 g). A fraction (95 mg) recovered from the zone with R_f 0.40 (Cf. cycloartenol, R_f 0.33; cholesterol, R_f 0.15) on TLC of the USM (3 g) consisted

exclusively of an unknown triterpene alcohol, mp 103-105 C. The mp and TLC, and the following spectral data of the unknown triterpene alcohol were found identical with those of the hydrolysis product (I) of the authentic specimen of I-acetate: IR ν_{max} cm^{-1}: 3500 (OH), 3050, 840, 820, 815 (>C=CH–). MS *m/e* [relative intensity]: 426.3878 [8, $C_{30}H_{50}O$, calcd. MW 426.3859], 411 [6], 408 [7], 274 [100], 259 [60], 231 [10], 205 [13], 163 [28], 161 [24], 152 [13], 137 [24], 134 [60], 123 [42], 121 [31], 119 [37], 107 [28]. ^{1}H-NMR:δ 0.80 (3H, *s*, C-13β*Me*) [normalized ΔEu, 29], 0.85 (3H, *s*, C-14α*Me*) [11], 0.92 (3H, *s*, C-9β*Me*) [32], 1.02 (3H, *s*, C-4α*Me*) [56], 1.13 (3H, *s*, C-4β*Me*) [100], 1.60 (3H, *s*, 26*Me*) [4], 1.68 (3H, *s*, 27*Me*) [3], 0.90 (3H, *d*, 21*Me*, *J*=5.9 Hz) [8], 3.49 (1H, broad *s*, 3α*CH*, $W_{1/2}$=5 Hz) [>200, undetermined], 5.09 (1H, *t*, 24*CH*, *J*=6.3 Hz) [5], 5.58 (1H, *d*, 6*CH*, *J*=5.9 Hz) [59].

Acetylation of the triterpene alcohol (55 mg) with acetic anhydride-pyridine at room temperature over night followed by crystallization gave the triterpene acetate (37 mg), mp 115-117 C (GLC, RRT: OV-17, 1.44; Poly I-110, 1.13). The mp and GLC data as well as the following spectral data of the triterpene acetate were identical with those measured for the authentic specimen of I-acetate: IR ν_{max} cm^{-1}: 1730, 1230 (OAc), 3050, 840, 820, 815 (>C=CH-). MS *m/e* [rel. int.]: 468.3965 [7, $C_{32}H_{52}O_2$, calcd. MW 468.3964], 453 [4], 408 [20], 393 [9], 355 [3], 297 [3], 274 [100], 259 [50], 231 [11], 205 [13], 163 [30], 161 [23], 150 [21], 137 [23], 136 [24], 134 [43], 123 [41], 121 [23], 119 [30], 107 [20]. ^{1}H-NMR:δ 0.81 (3H, *s*, C-13β*Me*) [normalized ΔEu, 9], 0.85 (3H, *s*, C-14α*Me*) [5], 0.91 (3H, *s*, C-9β*Me*) [17], 1.04 (6H, *s*, C-4α*Me*, C-4β*Me*) [C-4α*Me*, 19] [C-4β*Me*, 45], 1.59 (3H, *s*, 26*Me*) [1], 1.68 (3H, *s*, 27*Me*) [1], 2.01 (3H, *s*, C-3βO*Ac*) [100], 0.89 (3H, *d*, 21*Me*, *J*=6 Hz) [2], 4.70 (1H, *t*, 3α*CH*, *J*=2.5 Hz) [145], 5.09 (1H, *t*, 24*CH*, *J*=6.8 Hz) [1], 5.51 (1H, *d*, 6*CH*, *J*=5.9 Hz) [27].

RESULTS AND DISCUSSION

The triterpene alcohol reisolated here from the USM of gourd seed oil was reasonably identified as 10α-cucurbita-5,24-dien-3β-ol (anhydrolitsomentol, I) by the comparisons of mp and some chromatographic and spectroscopic data with those of the authentic specimen of I on both the free alcohol and its acetate. Assignments of the individual proton signals in the ^{1}H-NMR spectroscopy were achieved by a series of careful experiments with a lanthanide shift reagent in which the spectra were measured with every amount of $Eu(dpm)_3$ added, and then by the estimation of approximate spatial distance between the expected coordinating site (oxygen-containing function) of a lanthanide ion and the protons under consideration (4).

Various cucurbitacins, highly oxygenated tetracyclic triterpenes having a cucurbitane skeletone and a wide range of biological activities (5), are known to occur mainly in plants belonging to the Cucurbitaceae family and several other flowering plants (5-8). The triterpene I thus isolated from gourd seed oil is considered to be a representative parent compound of cucurbitacins, or an important intermediate in the biogenesis of the highly oxygenated triterpenes. The previously known simplest member of cucurbitane triterpene was litsomentol (2), a dihydroxy compound isolated from the bark of *Litsea tomentosa* (Lauraceae), from which the authentic specimen of I was derived by dehydration (2). Since the chemical dehydration of litsomentol could only be achieved under somewhat drastic condition, I from gourd seed oil is regarded indeed as the natural product rather than the artefact produced from litsomentol, the occurrence of which in the seed oil is highly probable, during the isolation procedure. Chromatographic evidences show that I occurs further in the other Cucurbitaceae seed oils from watermelon (*Citrullus Battich*) (1), snake gourd (*Trichosanthes Kirilowii*) (1) and pumpkin (*Cucurbita pepo*) (9).

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METHODS

A Method for the Quantitative Estimation of Cholesterol α-oxide in Eggs

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ABSTRACT

A method for the quantitation of cholesterol α-oxide in egg and egg products is described. Total lipids extracted from dry egg samples were fractionated on a silicic acid column to concentrate cholesterol oxides which were then quantitatively determined by gas liquid chromatography (GLC). Those samples which showed cholesterol oxides by GLC were further analyzed by high pressure liquid chromatography (HPLC) for the ratio of cholesterol α-oxide and cholesterol β-oxide. Cholesterol α-oxide content was calculated from the combined results of GLC and HPLC.

INTRODUCTION

In the presence of molecular oxygen and light, cholesterol autoxidizes to form chemically labile hydroperoxides which decompose into secondary products of which more than 20 have been identified or tentatively identified by previous workers (1-5). 5,6α-Epoxy-5α-cholestan-3β-ol (cholesterol α-oxide), one of the known cholesterol oxidation products, was first reported by Fioriti and Sims (4) in heat-treated (82 C) cholesterol and in samples which had been stored in air for several years. Since cholesterol α-oxide has been reported to induce tumor formation in rats and mice after subcutaneous administration by both oil and aqueous vehicles (6), and to be present in ultraviolet irradiated human skin (7), and in the skin of hairless mice (8), it was suspected to be carcinogenic. However, since cholesterol α-oxide responded negatively to the Ames mutagenicity screening test (9), it would only be indirectly involved in carcinogenesis if it is carcinogenic at all.

Cholesterol α-oxide was hydrolyzed in the gastrointestinal tract to 5α-cholestane-3β,5,6β-triol (10) which induced atherosclerotic lesions in rabbits in feeding studies (11) and also, in in vitro studies, caused acute cell death of the rabbit's aortic smooth muscle (12). Gray et al. (13) reported the findings of 1,000-4,000 μg/dl of cholesterol α-oxide in the sera of hypercholesterolemia patients but not in those of normal persons. These findings and the putative carcinogenecity of cholesterol α-oxide have prompted Benditt (14) to suggest that cholesterol α-oxide may play a role in the initiation of atherosclerotic lesions.

Despite its potential physiological effects, cholesterol α-oxide in foods has not been examined except for the report by Chicoye et al. (15) on the isolation of β-isomer, 5,6β-epoxy-5β-cholestan-3β-ol from spray-dried egg yolk exposed to sun and fluorescent lights. However, the failure to detect cholesterol α-oxide could be attributed to the insensitivity of experimental methods since β- and α-isomers were found to be present in the ratio of 8-11 to 1 in autoxidized cholesterol (16).

In all the previous studies, gas liquid chromatography (GLC) was used exclusively for the determination of cholesterol oxides despite its incapability of resolving α- and β-isomers in a mixture. Smith and Kulig (16) found that it was necessary to reduce the α- and β-oxides with lithium aluminum hydride to 5α-cholestane-3β,5-diol and 5α-cholestane-3β,6β-diol, respectively, and resolve the dihydroxy-derivatives by thin layer chromatography (TLC) and GLC. Due to the lack of recovery data and their laboriousness, none of the reported procedures is readily adapted to the analysis of cholesterol α-oxide in foods.

The method described below allowed us to determine cholesterol α-oxide directly in a relatively short time. It was developed using dried eggs as specimens for the obvious reason that eggs have the highest cholesterol content of all food systems.

MATERIALS

Cholesterol α-oxide (M.P. 137-139 C) was purchased from Steraloids Inc., Wilton, NH. The product was found by high pressure liquid chromatography (HPLC) analysis to contain 5% β-oxide and was free from other contaminants. Cholesterol β-oxide was prepared by the

method reported by Chicoye et al. (5) and purified with HPLC. Hexane, ethyl ether and chloroform, were glass-distilled grade as they were purchased from Burdick and Jackson, Muskegon, MI. Methanol was glass distilled by ourselves.

Silicic acid, Bio-Sil HA (minus 325 mesh), was the product of Bio-Rad Laboratories, Richmond, CA.

Extraction of Lipids

Dry egg sample (yolk, whole egg or egg blends) equivalent to 1 g of yolk solids was transferred to a 250 ml glass centrifuge bottle and 100 ml of chloroform/methanol (2:1, v/v) was added. The mixture was blended with a Polytron (PCU-2 Kinematica GmbH, Lucerne, Switzerland) at speed 6 for 30 seconds and then filtered through a Millipore PTFE filter (0.5 μM pore size) into a 250 ml evaporation flask. The residue was washed several times with a total of 20 ml solvent. The combined filtrate was dried under vacuum with a rotary-evaporator. The lipid extracts were redissolved in 5 ml of hexane/ethyl ether (95:5, v/v).

Enrichment of Cholesterol Oxides

A 1-g silicic acid column in a 5 ml graduated disposable glass pipet was dry packed. Its outlet was plugged with glass wool and the top surface protected with a layer of anhydrous sodium sulfate. The lipid extract was transferred to the column which was first wetted with 2 ml of the same solvent. Then a total of 15 ml solvent in 3 x 5 ml aliquots was used to rinse the flask and applied to the column. The column was then eluted with 30 ml hexane/ethyl ether (90:10, v/v) and 20 ml hexane/ethyl ether (50:30, v/v). Twenty ml of the eluent was collected after the elution was changed to the latter solvent.

Egg lipids contain ca. 65.5% triglycerides, 28.3% phospholipids and 5.2% cholesterol (17). Triglycerides and the majority of cholesterol were eluted by the 30 ml hexane/ethyl ether (90:10, v/v). Phospholipids remained in the column. The 20 ml eluent collected contained all recoverable cholesterol oxides, some cholesterol and a host of other unknown compounds, none of which interfered with the quantitation of cholesterol oxides in GLC and HPLC analysis. The eluents were dried under nitrogen stream and redissolved in 50 μl chloroform in 1 ml conical vial.

Quantitation of Cholesterol Oxides by Gas Liquid Chromatography

A Hewlett Packard Model 5830A with dual flame ionization detectors and dual nickel columns (2.1 mm ID x 2 *M*) packed with 3% OV-17 on 100/120 mesh Gas Chrom Q was used. Carrier gas, nitrogen, was delivered at a rate of 31 ml/min. Oven temperature was programmed from 225 to 260 C at 4 C/min. and isothermal at 260 C to completion. Injection port and detector temperatures were 270 and 325 C, respectively.

One μl of the chloroform solution was injected into the GLC. The peak appearing within ± 4% of the retention time of the standard cholesterol α-oxide was integrated. Peak area was converted to quantity of cholesterol oxides by the external standard method (18). The relative retention times of cholesterol α-oxide and its β-isomer with respect to cholestane were 3.17 and 2.98 and their mixture was unresolved by columns packed with OV-17, SE-30 or QF-1 as the liquid phase (5).

The FID detector did not respond linearly to concentration of cholesterol oxide from 0 to 1,000 ng/μl (Fig. 1). Therefore, the external standard used for any given sample should be as close to the concentration in the sample as possible. This sometimes required repetitive chromatographs. The lowest detectable concentration of cholesterol α-oxide was 50 ng/μl.

Determination of the Ratio of Isomers of Cholesterol Oxides by HPLC

A Waters Associates Model ALC/GPC-244 Liquid Chromatograph with Differential Refractometer (Model R401) and a μ-Porasil column (3.9 mm ID x 30 cm) was used. The mobile phase was 3 ml 2-propanol in 100 ml hexane, and the flow rate was 1 ml/min. A Perkin Elmer Model I computing integrator interfaced with the detector was used to detect and integrate peak area and carry out mathematical conversion.

Figure 2 shows the separation of cholesterol (II), cholesterol α-oxide (III) and its β-isomer (IV) in a synthetic mixture and in a typical cholesterol oxide fraction of lipids from a spray-dried egg product containing cholesterol oxides. The complete resolution of the isomers and that from the other unidentified peaks made the quantitation of cholesterol oxides possible. The relative retention volumes of cholesterol oxides to cholesterol are 3.70 for α-oxide and 4.43 for β-oxide. The lowest detectable level of the Differential Refractometer was 5 μg, which was 100-fold greater than that of the GLC. The peak area responded linearly to the injected sample from 50 μg to 300 μg.

Calculation of Cholesterol α-Oxide Content in Egg Products

The quantity of cholesterol α-oxide in dry

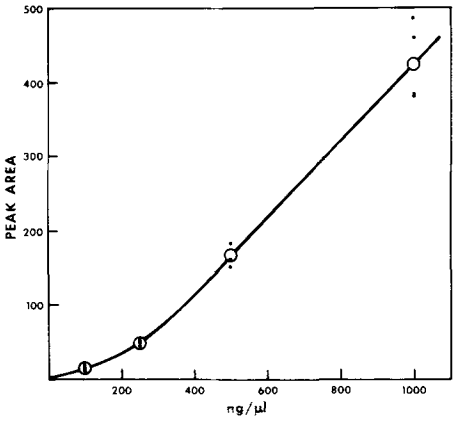

FIG. 1. Calibration of cholesterol oxide concentration and peak area in GLC analysis. The peak area was represented by an arbitrary unit preset in the integrator by the manufacturer.

egg products may be calculated as follows:

$$C = (50G/W)(a/(a+b))$$

where C: cholesterol α-oxide concentration in egg solids in ppm; G: cholesterol oxide concentration, including both α- and β-isomers, in solution analyzed by GLC in μg/μl; W: weight of egg solid in gm; a/(a+b): fraction of cholesterol α-oxide in cholesterol oxide mixture.

Let R = b/a: the ratio of cholesterol β-oxide to cholesterol α-oxide as determined by HPLC. Then,

$$C = 50G/W(1+R) \qquad 1.$$

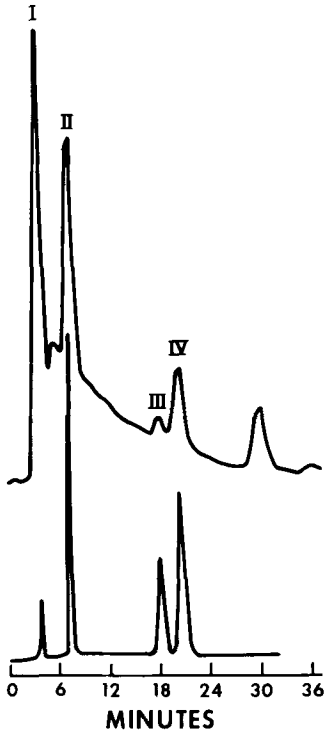

FIG. 2. HPLC separation of cholesterol (II), cholesterol α-oxide (III), and cholesterol β-oxide (IV) in a synthetic mixture (bottom chromatogram) and in a typical cholesterol oxides fraction of egg lipids (top chromatogram). See text for the condition used. Peak I was chloroform (solvent) and the other unmarked peaks were not identified.

RESULTS AND DISCUSSION

The efficiency of recovering cholesterol oxides from dried eggs was determined with a series of replicates of freeze-dried fresh yolk containing 50 and 200 ppm added cholesterol oxides. Three of the four control samples showed definitely no cholesterol oxides. The chromatogram of the fourth sample had a minor cholesterol oxide peak which amounted to less than 0.25 ppm. Since the confidence of GLC analysis was 2.5 ppm cholesterol oxides in egg solid, the finding was considered to be negligible.

Each freeze-dried yolk spiked with 50 ppm of cholesterol oxides was extracted in triplicate. Each extract was chromatographed in triplicate by GLC. The mean of the recovery of the nine analyses was 102% with coefficient of variance, 30%. The average recovery of 11 analyses of yolk containing 200 ppm of cholesterol oxides was 113% and with a coefficient of variance of 7%.

Since the reproducibility of external standard method for converting peak area to concentration relies heavily on injection technique, the precision of the GLC analysis was determined by injecting consecutively eight 1 μl of cholesterol oxides solution (500 ng/μl). The coefficient of variance was 11% which was comparable to that found in yolk solids containing 200 ppm cholesterol oxides. The coefficient of variance of the yolk spiked with low cholesterol oxides was obviously the result of combined effects of GLC analysis, lipids extraction and cholesterol oxides fractionation.

The coefficient of variance of HPLC determined by injecting repetitively 5 or 100 μg cholesterol α-oxide were 15% and 3%, respectively. However, reproducibility of HPLC had very little effect on the quantitation of cholesterol α-oxide, since only the ratio of α- and β-isomers was sought from each chromatogram (see Equation 1). The inconsistency which may occur due to the variation in sample size, environmental condition, etc. should have identical effect on both α- and β-isomers. In

practice, the replicates used in GLC analysis were combined, concentrated and analyzed by HPLC to partially compensate for its low sensitivity.

Cholesterol is a lesser component of the total lipids in most foods. The level of cholesterol α-oxide in foods is expected to be low, since it is only a minor component of the oxidation products of cholesterol (4), though its potential physiological importance is not to be minimized. The difficulties of quantitating a minor component in a complex natural product lie largely in eliminating the compounds which interfere with the quantitation. Saponification is commonly used for concentrating sterols from total lipids extracts and was used by Fioriti and Sims (4), Gray et al. (13) and Chicoye et al. (15), in their work. During saponification, glyceryl lipids are hydrolyzed by heated alkaline-alcohol solution and separated from sterols by aqueous extraction under alkaline condition (19). The highly strained three-membered epoxide ring of cholesterol oxides is likely to undergo a nucleophilic substitution reaction resulting in opening the ring (20). A known quantity of cholesterol α-oxide was saponified according to standard AOCS method, and only 25% of the original amount was recovered, Table I; this indicated that saponification should definitely be avoided in any quantitation procedure.

Table I also showed the effect of solvent on GLC analysis. Chloforom and tetrahydrofuran appeared to be the best solvents from a recovery standpoint; however, chloroform can corrode detector parts in extended use. When methanol was used in combination with chloroform, the recovery was reduced as the proportion of methanol increased. Acetone was also found unsatisfactory. If the cholesterol in the methanolic solution was held at room temperature for several days, then dried, redissolved in chloroform and analyzed by GLC, there was no loss in recovery. Therefore, the reaction between methanol and cholesterol oxides must occur at the injection port where the temperature was above 270 C.

CONCLUSION

Quantitation of cholesterol α-oxide in eggs was achieved because of two major factors: 1) This very minor component of the total lipids extracts of eggs was successfully concentrated with a preparative silicic acid column so that it could be subsequently separated from interfering compounds by GLC and HPLC; and, 2) the α- and β-isomers of cholesterol oxides were completely resolved by HPLC. The adaptation of the present method to other food or biological systems will depend on the nature of the possible interfering compounds and may require modification of the preparative silicic acid chromatography.

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TABLE I

Effects of Injection Solvents and Saponification on the Recovery of Cholesterol α-Oxide in GLC Analysis

Solvent used	Recovery %	V[a] % (replicates)
Chloroform	100	19 (16)
Chloroform/Methanol		
(95:5, vol.)	75	7 (3)
(67:33, vol.)	60	27 (4)
(50:50, vol.)	46	46 (3)
Acetone	49	10 (5)
Tetrahydrofuran	106	18 (8)
Chloroform (saponified)[b]	25	15 (4)

[a]V: coefficient of variance.

[b]Saponification was carried out according to AOCS Method Tk 1a-64T.

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COMMUNICATIONS

Distribution of Neutral Lipids in the Tissues of the Oyster *Crassostrea virginica*

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ABSTRACT

The content of neutral lipids was determined in the tissues of oysters (*Crassostrea virginica* Gmelin) collected in June, July and March. The lipid content of starved March oysters was also determined. Oyster tissue from the June harvest contained the highest quantities of triglyceride; starved and July (late spawning) oysters had decreased levels of triglycerides in all tissues except the digestive diverticula/gonad. Free sterol content of all the tissues averaged 1.21 mg equivalent cholesterol/g wet weight tissue, and the steryl ester concentration averaged about 10% of this value. Findings of this investigation indicate that the triglyceride content of oyster tissue fluctuates seasonally and is therefore keyed to the physiological state of the animal. Furthermore, triglycerides may be an important energy reserve for reproductive tissue.

INTRODUCTION

Glycogen, because of the large quantity stored, is believed to be the major metabolic fuel reserve for adult bivalve molluscs (1,2). Indeed, during periods of starvation or hibernation, the amount of glycogen changes more than any other tissue constituent (3,4).

The importance of lipids in the metabolic economy of molluscs is only now being appreciated. It has been calculated, by noting changes in body composition, that both mature Pacific oysters, *Crassostrea gigas*, (3) and immature European oysters, *Ostrea edulis*, (5) may generate more calories from lipids than other reserves. Further, lipids appear to serve a unique role in maturing molluscan gonadal tissue, as the females of some molluscan species contain more total body lipid than the males. After spawning begins, the lipid content of both males and females declines rapidly (6-8).

The present report concerns itself with the neutral lipid content of the American oyster, *Crassostrea virginica* Gmelin, in four physiological conditions. By providing data on the changes in tissue content of the individual classes of lipids, it is hoped that a better understanding of the role played by lipids in the overall metabolism of oysters may be gained.

MATERIALS AND METHODS

Oysters (3-5 cm in height and 3-4 cm long) were obtained from a commercial source (Chesapeake Oyster Culture Co., Shadyside, MD) in late March, early June, and late July. Oysters obtained in March were divided into two groups. One group of oysters (starved) was held in the laboratory in 12 parts per thousand artificial sea water ("Instant Ocean," Aquarium Systems, Inc., Eastlake, OH) at 18 C (9) for 37 days. During this time, no attempt was made to feed the animals; the saline was changed daily. The rest of the March oysters (normal) and all of the June (collected at or near the beginning of the spawning season – early spawning) and July oysters (collected towards the end of the spawning cycle – late spawning) were sacrificed immediately.

Tissues from the four groups were treated as follows. The tissues from three to six animals (either whole oysters or animals separated into mantle and gills, adductor muscle, and digestive diverticula/gonads) were drained on filter paper for several minutes. Each group of tissues was then extracted for lipids according to Folch et al. (10) with the aid of a Sorvall Omni-Mixer (Dupont Instruments, Newtown, CT). Upon completion of the Folch wash, the pooled crude lipid extracts representing three to six oysters were reduced in volume and stored in chloroform/methanol (2:1, v/v) at -10 C until analyzed.

Aliquots of the crude lipid extracts were separated on 0.25 mm silica gel 60 precoated thin layers without fluorescent indicator (EM Laboratories, Inc., Elmsford, NY) using *n*-hexane/diethyl ether/acetic acid, 90:10:1, v/v/v (11). The bands were visualized by I_2 vapor retention or under ultraviolet light after spraying the plate with 0.2% 2,7-dichlorofluorescein in ethanol. Silica gel containing the lipid bands of interest was scraped off the plate.

The lipids were recovered by elution with thirty volumes of chloroform/methanol, 2:1, v/v. Aliquots of appropriate lipid fractions were analyzed in triplicate for triglycerides (12) or sterols (13,14). Triplicate values agreed to within 3%. The free sterol fraction, as obtained by thin layer chromatography (TLC), was also analyzed by gas liquid chromatography (GLC) on 1.5% SE-30 (15) or 3% SE-30 (16) using a Shimadzu Seisakusho Model GS-5A gas chromatography (American Instruments Co., Silver Spring, MD).

Lipid standards were obtained from the following sources: cholesterol, triolein, oleic acid, methyl oleate, ethyl oleate, cholesteryl oleate, 5α-cholestane, cholestanol, and coprostanol-Sigma Chemical Co. (St. Louis, MO); cetyl alcohol, octadecyl palmitate and n-octatriacontane-Analabs (North Haven, CT); β-sitosterol and campesterol - Applied Science Laboratories, Inc. (State College, PA).

RESULTS

The results of the analyses of oyster neutral lipid classes after isolation by TLC are reported in Table I. Early spawning (June) oyster tissue contains the highest levels of triglycerides, normal (March) oyster tissue a lower amount, and the starved and late spawning (July) oyster tissue the least amount. Regardless of physiological state of the tissues analyzed, the digestive diverticula/gonad is richest in triglyceride.

The distribution of total free sterols appears to be uniform in all tissues, averaging 1.21 mg equivalent cholesterol per gram wet weight tissue. Steryl ester content is low, averaging only ca. 10% of the total sterol value. Throughout the tissues of the late spawning (July) oyster, steryl ester content is the least, averaging 3.7% of the total sterol value. The greatest differences in steryl ester content appear in the extracts of the digestive diverticula/gonad where the early spawning (June), normal (March) and starved tissues contain ca. 25% of their sterols as the ester, but the late spawning tissue has only 2.8% of its sterols as the ester. The low steryl ester to free sterol ratio in oyster tissues was confirmed by examining the crude lipid extracts for total and free sterols. Estimates of free sterols averaged 1.26 ± 0.42 mg equivalent cholesterol/g wet weight in the crude extracts vs. 1.26 ± 0.37 mg equivalent cholesterol/g wet weight recovered after thin layer separations. Average values for steryl esters were 0.25 ± 0.12 mg equivalent cholesterol/g wet weight in the crude extracts as against 0.14 ± 0.18 mg equivalent cholesterol/g wet weight recovered after TLC.

GLC methods were employed in an attempt

TABLE I

Neutral Lipid Content[a] of Oysters in Four Physiological Conditions

Tissue	Condition	Free sterol	Steryl ester	Total sterol[b]	Triglycerides
		(mg Equiv.[c] cholesterol/g wet weight tissue)			(μg Glycerol/g wet weight tissue)
Total oyster	Normal (March)	1.13	0.14	1.27	930
	Early-spawning (June)	1.06	0.29	1.35	1432
	Late-spawning (July)	0.72	0.03	0.75	286
	Starved	1.58	0.22	1.80	420
Mantle and gills	Normal (March)	1.10	0.01	1.11	563
	Early-spawning (June)	1.23	0.13	1.36	911
	Late-spawning (July)	0.87	0.07	0.94	360
	Starved	1.31	0.23	1.54	358
Adductor muslce	Normal (March)	1.22	0.01	1.23	619
	Early-spawning (June)	0.83	0.07	0.90	226
	Late-spawning (July)	1.10	0.01	1.11	148
	Starved	1.69	0.03	1.72	450
Digestive diverticula and gonad	Normal (March)	1.21	0.31	1.52	992
	Early-spawning (June)	1.13	0.25	1.38	1879
	Late-spawning (July)	1.08	0.03	1.11	607
	Starved	2.10	0.63	2.73	1935

[a]Recovered after TLC.

[b]Sum of free sterol and steryl ester values.

[c]Digitonin precipitables.

to further characterize the free sterol fraction of oyster lipids. Results obtained using 1.5% or 3% SE-30 were comparable. At least eight sterols are found in oysters. From area percent calculations, it was determined that cholesterol, brassicasterol, and 24-methylenecholesterol, at 36.5%, 11.8% and 27.9%, respectively, constitute the bulk of the sterols. Minor sterol components found were: 6.27% 22-dehydrocholesterol, 3.2% campesterol, and three unidentified peaks of 4.3%, 9.5% and 0.6% each.

DISCUSSION

The seasonal variations of oyster glycogen content are well documented, especially the precipitous decline just after the onset of spawning (2). However, the variations among the classes of lipids in oyster have not been studied. Oysters under physiological stress show decreased triglyceride levels in all tissues except the digestive diverticula/gonad. This general decrease in tissue triglycerides indicates that the American oyster has the ability to mobilize both glycogen and triglycerides to meet its metabolic demands. In this regard it is like the Pacific oyster, *C. gigas* (3). The effect of prolonged storage at 2 C - 4 C in air on the lipids of adult living oysters has been reported (4,17). Under these conditions, the oyster is anaerobic and unable to catabolize lipids. Therefore, it is not surprising that the previous examinations of lipids in oysters under this type of physiological stress did not reveal marked decreases in lipid content.

The comparatively high levels of triglycerides in the digestive diverticula/gonad leads to a speculation that: (a) some portion of this tissue is a storage depot for oyster triglycerides; (b) some portion of this tissue is the major site for metabolism of triglycerides; (c) triglycerides may serve as a major energy reserve for the gonads; (d) this may be the pool from which triglycerides are withdrawn and redeposited in the maturing ova (the eggs of two other oyster species, *O. edulis* (5) and *C. gigas* (3), are rich in triglycerides). With reference to points c and d, it should be re-emphasized that the tissues were grossly dissected and that the gonads were taken along with the digestive diverticula. It has been suggested that some molluscs utilize fat as the fuel in their gonads (1,7). The present findings support this hypothesis. Furthermore, the seasonal shifts in composition and weight of the digestive gland of the clam, *Mytilus edulis*, suggest an energy storage role (18).

The literature presents conflicting and incomplete reports of oyster sterol composition. All reports, including the present study, utilized only one GLC phase. Conclusions concerning the identity of peaks under such circumstances must be viewed with caution. Comparison of the percentages of various sterols found in the present study with those reported in the literature (19,20) reveals close agreement. The predominate oyster sterols are cholesterol (35%), 24-methylenecholesterol (27%) and brassicasterol (12%).

The decrease in sterol constituents of the postspawning oyster tissues is probably due to the loss of these lipids during the reproductive process. Such losses in both free and esterified sterols have been noted in the scallop, *Placopectan magellanicus* (6). Conflicting values for the percent of concentrations of steryl esters to free sterols in oyster tissue have been published. Baker et al. (4) estimated this ratio to be almost 50%, while Watanabe and Ackman (21) found it to vary from 9.3% to 28.5%. The present report agrees with the latter study as values found herein for this ratio average 10% and vary from 1% to 30%.

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LETTERS TO THE EDITOR

Cholesterol Absorption

Sir: The methods of determining cholesterol absorption which are in use all require assumptions which make them impossible to interpret in absolute terms. Direct transfer of cholesterol to lymph is accurate, but its determination requires surgery which makes physiological interpretation difficult. Other methods resort to selected assumptions, and comparison of one method to another is not an indication of validity.

The first problem is transit time through the gastrointestinal tract. Various markers have been used to enable correlation of time of the period of ingestion of a particular diet with the excretion related to that time period. Chromic oxide has been validated as suitable for that purpose (1). The authors recommended that only subjects who excreted greater than 90% of the chromic oxide dose/day be included in balance studies. Twenty percent of their subjects sequestered the Cr_2O_3 in the colon. This obviously eliminates 20% of the "normal" population from any study.

The major problem is losses of neutral sterols during intestinal transit, which were considered to be unaccountable (2-3). Labeled β-sitosterol has been considered to be a suitable nonabsorbable marker for sterols. The conviction that β-sitosterol is not absorbed is based upon reports of Gould (4) for man and Sylven and Borgstrom (5) for the rat. In fact, Gould proved that β-sitosterol was absorbed by humans, incorporated into tissues, esterified and excreted via bile (4). The efficiency of absorption was ca. 10% of that of cholesterol in his subjects. Sylven and Borgstrom (5) showed 1.5 to 2.7% of fed sitosterol was transported to the lymph in the rat.

Other reports have indicated absorption of 22% (6), 2 to 23% (7), and 53% (8) of fed plant sterols by rats. These results were based on excretion of the apparently unabsorbed materials. Davignon et al. (1) reported failure of excretion of 8-43% of fed β-sitosterol by human subjects. The excretion pattern was not correlated with intestinal transit time.

The low concentration of β-sitosterol in plasma has been one reason absorption has been considered low. Salen et al. (9) concluded that ca. 10% of β-sitosterol was absorbed based upon measurements only of plasma concentration. It may be concluded that β-sitosterol disappears from the gut, enters the lymph, is incorporated into tissues and excreted in bile in varying proportions of what is ingested. The appearance of plant sterols in skin is further confirmation of their absorption (10).

The failure of loss of carbons from the ring structure of cholesterol was verified in 1952 by Chaikoff et al. (11) and has been reconfirmed by Chevallier (12). In fact, intestinal bacterial degradation and carbon loss does not occur (13,14). The germ-free baboon has been shown to excrete only about half the administered β-sitosterol determined either by recovery of mass or radioisotope (15). In that experiment, the fecal recovery of cholesterol was about the same as that of sitosterol, and neither was greatly affected by mono or polycontamination of the baboon's gastrointestinal tracts.

Use of β-sitosterol as a nonabsorbable marker is fallacious. The difference between ingested and excreted radiolabeled cholesterol is net absorption. What happens to the cholesterol which is not excreted must be accounted for. This use of radiolabeled cholesterol provides information on net absorption which adds to the information which may be derived from chemical balance methods. Still unaccounted for is the actual absorption and enterohepatic circulation of cholesterol when these methods are used.

The Plasma Isotope Ratio Method for determination of cholesterol absorption was proposed by Zilversmit for rats (16). It involves simultaneous administration of [^{3}H]cholesterol orally and [^{14}C]cholesterol intravenously (or vice versa) and subsequent analysis of the ratio of ^{14}C and ^{3}H in serum cholesterol.

$$\frac{\text{Percentage of the oral dose in an aliquot of plasma}}{\text{Percentage of the intravenous dose in the same aliquot of plasma}} \times 100$$

In rats, the ratio was constant for several days after the first 24 hr.

The plasma ratio is rationally sound. The intravenous injection of cholesterol results in 100% of the dose being within the animal. When the two isotopes disappear at similar rates, as demonstrated in the rat, one or two measurements constitutes as valid a measure of

absorption as is presently known. The ratio incontrovertibly indicates internal/external cholesterol. If the curves are not parallel, then one must make measurements until they become parallel and then calculate the area ratios, rather than the simple plasma ratio. Validity then becomes a question of mathematics rather than assumptions about losses of carbon.

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Analysis of Alpha Tocopherol in Red Blood Cells by Gas Liquid Chromatography

Sir: A previous communication (Lehmann, J., Lipids 13:616 [1978]) described a procedure for the analysis of α-tocopherol in plasma and platelets by gas liquid chromatography (GLC). When we tried to extend this methodology to the analysis of α-tocopherol in red blood cells, an interfering substance thus far encountered only with red blood cells was not separated adequately from the 5,7-dimethyltocol (5,7-T) internal standard on GLC (Fig. 1). The method now has been successfully adapted to the analysis of red blood cells by the following modification.

Nonsaponifiables from 0.5 ml of packed red cells were dissolved in 0.5 ml of toluene (toluene was purified before using by passing through 80-200 mesh neutral alumina) and passed through 0.3 g of toluene-washed Unisil® (100-200 mesh activated silicic acid, Clarkson Chemical Co., Williamsport, PA) in a Pasteur pipet. The column was then rinsed with 1.6 ml of toluene and the total effluent (2.1 ml) was collected in an evaporation tube, evaporated to dryness with nitrogen and prepared for GLC as described previously. Elution volume characteristics of different lots of Unisil® may vary and should be determined.

Recoveries of α-tocopherol relative to 5,7-T added to vitamin E-deficient rat red blood cells (N=5) averaged 89% (range 87-92%). The coefficient of variation for replicate analyses (N=10) was 4.42% ($\bar{x}$-1.94 μg α-tocopherol/ml packed cells). Red blood cells containing as little as 0.2 μg of α-tocopherol/ml packed cells have been analyzed successfully by using these modifications and without increasing sample size.

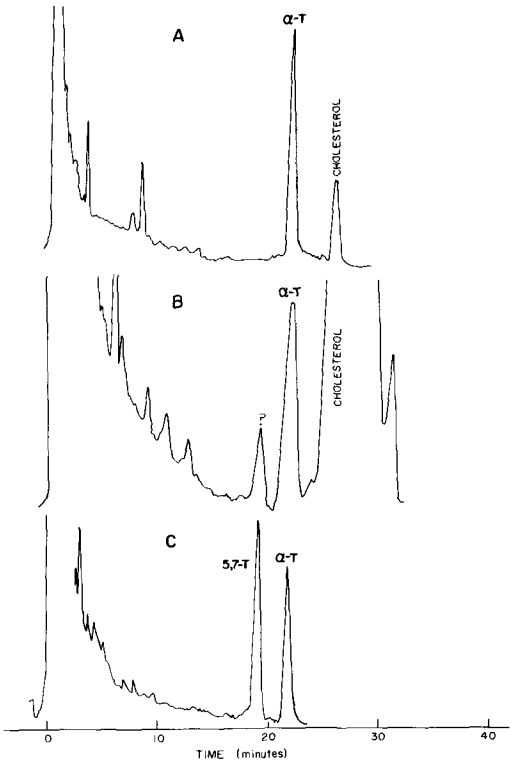

FIG. 1. Gas chromatographic separation of rat red blood cell α-tocopherol (A) after Unisil® column, (B) without Unisil® column, and of (C) a standard mixture of 5,7-dimethyltocol (5,7-T) and α-tocopherol (α-T). Column treatment also improved GC resolution.

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Coloring Conditions of Thiobarbituric Acid Test for Detecting Lipid Hydroperoxides

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ABSTRACT

The coloring reaction of the thiobarbituric acid test for hydroperoxides was completely inhibited by the addition of EDTA. Therefore, it was necessary to add a metal salt to the reaction mixture to complete the reaction and also to add an antioxidant to prevent autoxidation when unoxidized unsaturated fatty acids co-exist. The optimal pH of the reaction was found at 3.6 using glycine-hydrochloric acid buffer.

INTRODUCTION

Thiobarbituric acid (TBA) reaction has long been used to detect lipid oxidation(1). The TBA reaction has been considered to depend on the reaction forming red pigment by reacting with malonaldehyde produced by lipid oxidation, and generally used for detecting so-called secondary products (2). In recent years, this reaction has been used for the determination of hydroperoxides in living tissues (3,4). Therefore, basic studies using purified hydroperoxides are necessary to establish the TBA test for hydroperoxides applied to serum or some biological systems. In our previous paper (5), we proposed to use iron salt and an antioxidant to elevate the sensitivity of the method when the TBA reaction is applied to the determination of hydroperoxides. After that paper (5) was submitted, Ohkawa and Yagi (6) reported a highly sensitive method which uses a detergent and an acetate buffer, pH 4.0. Therefore, we again tried to find the best conditions for the TBA test by adopting the good points of their method. From the results, we realized that the TBA test for hydroperoxides depends on the catalytic action of contaminated metal ions in the reagent, because this coloring reaction was completely inhibited when EDTA was added. Therefore, the addition of a metal salt to the reaction mixture is necessary for completing the reaction. The best coloring was obtained by using glycine-hydrochloric acid buffer, pH 3.6, as determined by its high color formation and high color purity. Antioxidants were also indispensable to prevent autoxidation when unoxidized lipids co-existed.

MATERIALS AND METHODS

Materials

Methyl linoleate and methyl linolenate were purchased from the Tokyo Kasei Co. Fatty acid esters were dissolved in hexane and were purified through a Florisil column (7). Methyl linoleate monohydroperoxides (MLHPO) and methyl linolenate monohydroperoxides (MLNHPO) were purified by silica gel column chromatography from fatty acid methyl esters autoxidized at 37 C (8).

Butylated hydroxytoluene (BHT) was used as an antioxidant; ethanol solution was used.

Determination of hydroperoxides. Iodometric determination was used (9).

TBA test. A modified Ottolenghi method (10) was used. To 0.1 ml of the sample in a 20 ml test tube, 0.1 ml of 5% sodium dodecyl sulfate (SDS), 1.5 ml of acid or buffer solution and 1.5 ml of 0.5% TBA solution (depending on the experimental conditions, 0.1 ml of ferric chloride solution, $FeCl_3 \cdot 6H_2O$, 270 mg in 100 ml water and 0.1 ml of BHT ethanol solution, BHT 220 mg in 100 ml ethanol) were added. The tube was capped with a glass bead and the mixture was heated for 15 min in a boiling water bath. After it was cooled in ice water, 1 ml of glacial acetic acid and 2 ml of chloroform were added. The mixture was then shaken and centrifuged. The optical density of the supernatant was determined at 532 nm using a 1 cm cuvette. The final volume was ca. 4.2 ml.

A reagent blank was run simultaneously. The molar extinction coefficient used to calculate the amount of malonaldehyde was 1.56×10^5 M^{-1} cm^{-1} (11).

Final proposed TBA test. To 0.1 ml of the sample, 0.1 ml of ferric chloride solution (same as described above), 0.1 ml of BHT solution (same as described above), 1.5 ml of 0.2 M glycine-hydrochloric acid buffer, pH 3.6 (12), and 1.5 ml of TBA reagent were added. The procedures followed were the same as mentioned above. The TBA reagent was made by dissolving 0.5 g of TBA and 0.3 g of SDS in 100 ml water. The final volume was ca. 4.1 ml.

RESULTS AND DISCUSSION

Several kinds of buffer solution were tested for the coloring condition of hydroperoxides

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TABLE I

TBA Test for MLHPO and MLNHPO at Several pHs[a]

	Coloring (%)					
	35%[b] TCA	0.2 M acetic acid	0.2 M glycine-HCl, pH 3.6	0.2 M[c] acetate pH 4.0	0.2 M glycine-NaOH, NaOH, pH 8.8	0.01 M NaOH
MLHPO	11	58	100[d]	102	90	80
MLNHPO	20	64	103	89	90	75

[a]The reaction was carried out as described in "Methods." 0.5 μmole each of either MLHPO or MLNHPO was used per tube. One μmole ferric chloride was added to each tube. Mean values from triplicate experiments are used. The absorption maxima of the final solutions are the same in all cases.

[b]Ref. 5 and 10.

[c]Similar values were obtained from using 20% acetic acid adjusted with sodium hydroxide to pH 4.0 (6).

[d]25.0 nmoles malonaldehyde.

with TBA test. At the same time, the effects of ethylenediaminetetraacetic acid (EDTA) and iron salts were tested. The results showed that the TBA test for hydroperoxides was completely inhibited by EDTA but the so-called secondary products reacted in its presence. These facts show that the reaction when applied to hydroperoxides depends on the catalytic action of contaminated metal ions in the reagents. Tests were then conducted using iron salts. Ferrous sulfate was found to be effective when trichloroacetic acid (TCA) was used, as shown in our previous paper (5), but only ferric chloride was effective under other conditions; i.e., weak acid or alkaline pH. Therefore, 1 μmole ferric chloride per tube was used in the following experiments.

The TBA test was carried out at several pHs with the addition of ferric chloride. The TBA color could not be obtained when citrate or phosphate buffers were used. This fact may also support the necessity of iron salts to the reaction. In all cases, 15 min heating was enough to get maximum color. As shown in Table I, the color intensities were higher when glycine-hydrochloric acid buffer, glycine-sodium hydroxide buffer and acetate buffer were used. But the purity of the color obtained was more prominent in the cases of glycine-hydrochloric acid and glycine-sodium hydroxide buffers than that of acetate buffer. The color obtained using glycine-hydrochloric acid buffer, pH 3.6, was pure pink (532 nm) and did not contain a yellow fraction (about 450 nm) as shown in Figure 1. The color resulting from glycine-sodium hydroxide buffer, pH 8.8, was also pure pink.

The optimal pH conditions may be determined by two factors, the formation of coloring materials (e.g., malonaldehyde) from the decomposition of hydroperoxides, and the reaction of malonaldehyde with TBA. Both glycine buffers, pH 3.6 and 8.8, were considered to be suitable for the TBA test.

When unoxidized fatty acids co-existed in the reaction condition, autoxidation proceeded. To depress the autoxidation of unsaturated fatty acids, BHT was added. As shown in Table II, BHT was effective in depressing autoxidation. The addition of BHT, 1 μmole/tube, was adopted for the following experiments. As the decomposing reaction of hydroperoxides to malonaldehyde is a radical reaction, BHT partially inhibited the TBA reaction (Table III).

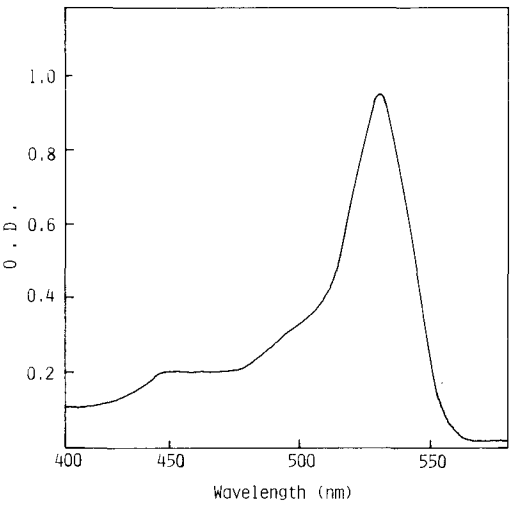

FIG. 1. Absorption spectrum of the color produced by the reaction of MLHPO with TBA. The reaction was carried out with 0.5 μmole MLHPO in glycine-hydrochloric acid buffer, pH 3.6. Ferric chloride 1 μmole was added. Test are described under "Methods."

TABLE II

Effect of BHT on the Autoxidation of Methyl Linoleate and Methyl Linolenate in Glycine-Hydrochloric Acid Buffer, pH 3.6, and Glycine-Sodium Hydroxide Buffer, pH 8.8.[a]

	Coloring (%)			
	Methyl linoleate		Methyl linolenate	
	pH 3.6	pH 8.8	pH 3.6	pH 8.8
+ $FeCl_3$ 1 μmole	100[b]	57	100[c]	75
+ $FeCl_3$ 1 μmole + BHT 1 μmole	0	0	5	4
+ EDTA 1 μmole	0	0	0	0

[a]The reaction was carried out as described under "Methods." Methyl linoleate and methyl linolenate used/tube were 50 μmoles and 5 μmoles, respectively. Mean values from triplicate experiments are used.

[b]21.8 nmoles malonaldehyde.

[c]93.2 nmoles malonaldehyde.

TABLE III

Effect of BHT on the Coloring of Hydroperoxides[a]

	Coloring (%)			
	MLHPO		MLNHPO	
	pH 3.6	pH 8.8	pH 3.6	pH 8.8
+ $FeCl_3$ 1 μmole	100[a]	90	104	91
+ $FeCl_3$ 1 μmole + BHT 1 μmole	54	53	63	61
+ EDTA 1 μmole	0	0	0	0

[a]MLHPO and MLNHPO used were all 0.5 μmole/tube. Glycine-hydrochloric acid buffer, pH 3.6, and glycine-sodium hydroxide buffer, pH 8.8, were used. Values are the means from triplicate experiments and are expressed as percentages of those obtained from MLHPO without BHT.

[b]25.0 nmoles malonaldehyde.

However, both a ferric salt and an antioxidant are necessary for the TBA test of hydroperoxides when unoxidized unsaturated fatty acid co-existed. An appropriate balance of ferric salt and BHT may be important. Autoxidation of unsaturated fatty acids proceeded faster at pH 3.6 than at pH 8.8, but it was almost prevented by the addition of BHT (Table II). The coloring of hydroperoxides was depressed by adding BHT at both pH 3.6 and 8.8, but without BHT, the coloring was a little higher at pH 3.6 (Table III). Therefore, glycine-hydrochloric acid buffer, pH 3.6, was finally chosen for the proposed method in this paper as described under "Methods."

From the results obtained above, it should be noted that the TBA test cannot be used for determining the quantity of hydroperoxides, but it can be used for obtaining the relative amount of hydroperoxides in a definite sample.

By using the TBA method described above, calibration curves of MLHPO and MLNHPO were obtained in the presence of unoxidized unsaturated fatty acids (Figs. 2 and 3). By adding methyl linoleate (Fig. 2), the coloring was a little lower than simply by adding BHT. With methyl linolenate (Fig. 3), the values of the curve obtained increased a little by containing a portion of the color derived from autoxidized linolenate. As shown in these calibration curves, only 5% of hydroperoxides was detected as malonaldehyde. One thing should be noted is that pure linoleate hydroperoxides formed TBA color against the results of Dahle et al. (13).

When the TBA test is applied to detecting hydroperoxides in a system containing both hydroperoxides and unoxidized lipids, ferric chloride and BHT should be added as shown above. When the TBA test is used for detecting only secondary products, either BHT or EDTA

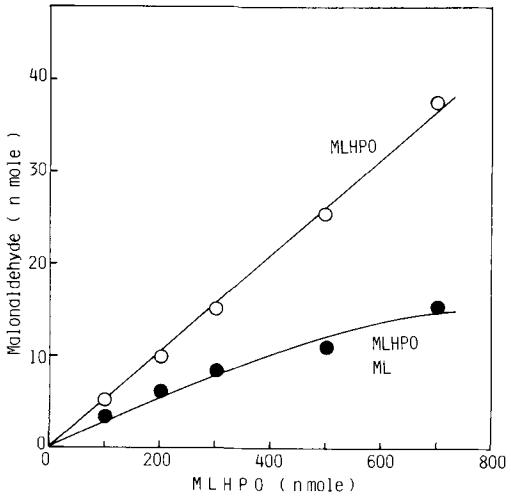

FIG. 2. Calibration curves of MLHPO with and without unoxidized linoleic acid. The TBA test was performed with 0.2 M glycine- hydrochloric acid buffer, pH 3.6.
—○—○— MLHPO only, tested according to our final proposed method except BHT was not added.
—●—●— MLHPO with 50 μmoles linoleic acid, tested according to our final proposed method.

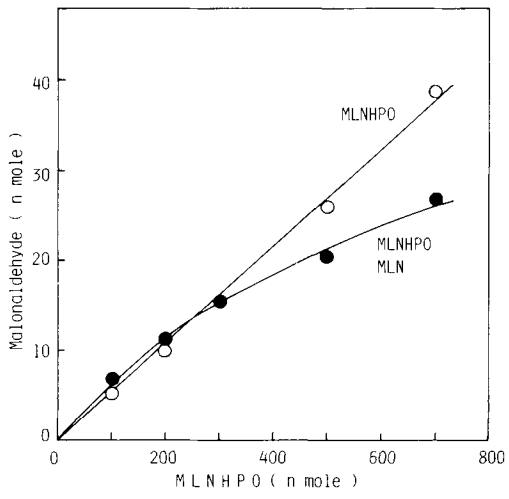

FIG. 3. Calibration curves of MLNHPO with and without unoxidized linolenic acid. The TBA test was performed with 0.2 M glycine-hydrochloric acid buffer, pH 3.6.
—○—○— MLNHPO only, tested according to our final proposed method except BHT was not added.
—●—●— MLNHPO with 5 μmoles linolenic acid, tested according to our final proposed method.

should be added.

This method is not considered to be appropriate to applying any materials, but may be useful for the microdetermination of hydroperoxides in serum or so.

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Effect of Methyl 2-Hexadecynoate on Hepatic Fatty Acid Metabolism

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ABSTRACT

Normal and hepatoma bearing rats were fed a low level of methyl 2-hexadecynoate in a low fat diet for one month. The effect of the acetylenic acid on lipid metabolism as derived from mass analysis of lipid classes, fatty acids and positional monoene isomers isolated from the major lipid classes of liver and hepatoma has been assessed. Methyl 2-hexadecynoate caused a 25% decrease in body weight and the appearance of essential fatty acid deficiency symptoms within one week. Non-tumor-bearing animals contained a seven-fold increase in all neutral lipid classes, except cholesterol, while host animals did not contain fatty livers. The apparent protective effect of the host animal by the hepatoma also resulted in only marginal changes in the fatty acid and positional monoene isomers from host liver and hepatoma lipids. In contrast to host liver and hepatoma, methyl 2-hexadecynoate caused a massive accumulation of palmitate and hexadecenoates with a concomitant decrease in stearate and octadecenoates in most of the lipid classes from non-tumor-bearing animals. These changes were accompanied by a shift from the higher molecular weight triglycerides to lower molecular weights corresponding to carbon number 48. The high concentrations of hexadecenoates consisted predominantly of the $\Delta 9$ isomer. Despite the high concentrations of *cis* $\Delta 9$ hexadecenoate, precursor of *cis* $\Delta 11$ octadecenoate (vaccenate), total vaccenate levels of the five major lipid classes were lower than control values. All of these data strongly suggest that long-chain 2-ynoic acids inhibit elongation of saturated and monoene fatty acids.

INTRODUCTION

We have made use of a number of compounds that affect lipid metabolism to study the relation between hepatoma and host animal lipids (1-3). An acetylenic fatty acid, 5,8,11,14-eicosatetraynoic acid, exhibited multiple effects on liver lipids, and to a lesser degree the hepatoma lipids (3), and appeared to retard the rate of hepatoma growth (unpublished data). Because of the poor solubility and apparent low absorption of 5,8,11,14-eicosatetraynoic acid, a more effective acetylenic acid was sought. This report describes the effect of methyl 2-hexadecynoate on the lipid metabolism of normal liver, host liver and hepatoma 7288CTC.

METHODS AND MATERIALS

Groups of male Buffalo strain rats weighing 250-300 g were placed on a fat-free diet (U.S. Biochemical Corp., Cleveland, OH) supplemented with 0.5% safflower oil or supplemented with 0.5% safflower oil plus 0.5% methyl 2-hexadecynoate. The level of the methyl 2-hexadecynoate was reduced to 0.1% after one week. A group of rats on the methyl 2-hexadecynoate diet was transplanted with hepatoma 7288CTC at the time the animals were placed on the diet. After four weeks, the animals were killed, livers and hepatomas excised, weighed, lyophilized and the total lipids extracted by the Bligh and Dyer procedure (4). Neutral lipids and polar lipids were fractionated by silicic acid chromatography (5). Neutral lipid classes were quantified by high temperature gas-liquid chromatography (GLC) analysis of the intact lipids (6). Phospholipid classes were resolved by thin-layer chromatography (TLC) (7) and quantified by phosphorus analysis (8). Individual lipid classes were isolated by TLC (6,7), methyl esters prepared by acid catalyzed esterification (6) and analyzed quantitatively by GLC on 10% SP-2330 (Supelco, Inc., Bellefonte, PA) columns programmed from 140 C to 220 C at 3 C/min using a Varian Model 3700 chromatograph. Fatty esters were resolved according to degree of unsaturation and configuration on Silica Gel G Plates impregnated with 6% silver nitrate and developed in chloroform containing 1% ethanol. The *cis* monoene fraction was resolved by preparative GLC (9) on a nonpolar column and the hexadecenoate and octadecenoate fractions were collected (10). Ozonides of the monoenes were prepared by a modification of the Beroza and Bierl procedure (11,12) and analyzed by GLC (11). All GLC analyses were taken with a Spectra-Physics digital integrator (Santa Clara, CA).

The 2-hexadecynoic acid was prepared by the addition of carbon dioxide to the Grignard of 1-pentadecyne. The methyl ester gave only one spot on TLC. Analysis of the methyl

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ester by GLC on polar (SP-2330) and nonpolar (OV-1) columns gave only one peak having an equivalent chain length of 19.4 and 16.6, respectively, on the two columns. After hydrogenation, only one peak was observed on the two columns corresponding to methyl palmitate. Ozonolysis of methyl hexadecenoate, resulting from partial hydrogenation of methyl 2-hexadecynoate, gave only myristaldehyde confirming the position of the triple bond. The source and purity of solvents and standards used in these studies have been reported (11).

RESULTS

Effect on Intact Animal

The methyl 2-hexadecynoate had an immediate and dramatic effect on the rats. By the second day on the diet, the animals had red, irritated eyes, noses, mouths and front paws. The irritation may have been produced directly by the acetylenic acid or by continued grooming and washing of the face. The face and paws were always wet, which probably resulted from nasal weeping or salivation. After a few days the hair on the face and paws became a dark brown to red which was assumed to result from blood. Within a week the animal had the appearance of severe essential fatty acid deficiency: the paws and face became scaly and the hair coat over the whole body was rough. These essential fatty acid deficiency symptoms may have resulted from other causes (hepatotoxicity) since the level of 18:2 and 20:4 in the liver lipids appeared normal. This does not preclude the possibility that the metabolism of the essential fatty acid or derived products was inhibited.

Body weight decreased approximately 25% in one week for normal and hepatoma bearing animals being fed the 0.5% methyl 2-hexadecynoate in the diet. Because it appeared the animals would die soon, the amount of methyl 2-hexadecynoate was reduced to 0.1% of the diet for the remaining three weeks. The animals then maintained their weight the second week and gained weight the third and fourth weeks, reaching 87% of their original weight, but the appearance of the essential fatty acid deficiency persisted. Control animals gained 50 to 60 g during the experiment.

Lipid Classes

Liver weights (5.6% ± 0.3), expressed as percent of body weight, of normal animals fed the acetylenic acid were significantly higher than host liver (4.6% ± 0.3 of rats fed methyl 2-hexadecynoate or normal liver (4.6% ± 0.04) of the untreated animals. The quantity of total neutral lipids, total phospholipids, and the percentage distribution of the individual lipid classes in each fraction are given in Tables I and II. Neutral lipid levels for control liver, host liver and hepatoma were similar to normal liver, host liver and hepatoma from chow fed animals reported previously (13). In contrast, normal liver from animals fed the acetylenic acid contained more than seven times the neutral lipids of control liver. Despite the high concentration, the percentage distribution of the lipid classes was not very different from control liver with the exception of the lower percentage of cholesterol (Table I). Generally, free fatty acids, cholesterol and cholesteryl esters were higher and triglyceride percentages were lower in host liver and hepatoma compared to control liver and liver from methyl 2-hexadecynoate fed animals. A comparison of the distribution of neutral lipid classes with values reported previously (13) for chow fed animals indicates the acetylenic acid lowered cholesterol and triglyceride levels in host liver and the hepatoma. Lysophosphatidylcholine and phosphatidylserine percentages were elevated in the liver of non-tumor-bearing animals fed the acetylenic acid. Generally, the effects of methyl 2-hexadecynoate on liver phospholipid concentrations and phospholipid class distributions were minimal (Table II) and values agreed with those reported earlier for chow fed animals (14). The lower concentration of phospholipids, the elevation of sphingomyelin and lower percentage of phosphatidylcholine in the hepatoma relative to liver have been observed earlier (14) and do not result from the acetylenic acid.

The carbon number distribution of the triglycerides from hepatoma and liver of animals fed the acetylenic acid is given in Table III. Liver from each group of animals and hepatoma exhibited a different distribution. Both host liver and normal liver of the animals fed the acetylenic acid contained a high percentage of carbon number 48 and a much reduced percentage of carbon number 52 relative to normal liver of untreated animals. The triglyceride carbon number distribution of control liver was intermediate to triglycerides from animals fed chow and fat-free diet (13). The higher molecular weight triglycerides of the hepatoma differed little from hepatomas obtained from host animals fed chow or fat-free diets (13). The higher percentage of high carbon number triglycerides is not unique to hepatomas, but is characteristic of a large number of transplantable rat and mouse tumors (15).

TABLE I

Percentage Composition of Neutral Lipids from Hepatoma and Liver of Animals Fed Methyl 2-Hexadecynoate

Hepatic tissue and diet	Quantity NL (mg/g wet wt)	Lipid Class Percentages[a]				
		FFA	Chol.	DG	TG	CE
Control liver[b]	22.7 ± 3.7	2.6 ± 1.2	7.8 ± 1.2	4.8 ± 0.6	82.0 ± 1.7	2.8 ± 0.5
Normal liver, treated[c]	174.1 ± 22.4	3.6 ± 3.1	1.1 ± 0.3	3.5 ± 1.3	87.6 ± 5.0	4.2 ± 0.7
Host liver, treated	7.2 ± 0.6	21.8 ± 4.5	23.0 ± 3.5	7.8 ± 1.3	38.8 ± 1.6	7.6 ± 2.4
Hepatoma, treated host	7.0 ± 1.5	5.3 ± 1.4	25.2 ± 6.2	0.9 ± 0.5	41.6 ± 9.5	24.7 ± 8.7

Abbreviations: NL = total neutral lipid; FFA = free fatty acids; Chol. = cholesterol; DG = diglycerides; TG = triglycerides; and CE = cholesterol esters.

[a]Percentages represent the mean ± standard deviation of three or four animals.

[b]Control diet consisted of fat-free + 0.5% safflower oil.

[c]Treated diet consisted of control diet + 0.1% of methyl 2-hexadecynoate.

TABLE II

Percentage Distribution of Phospholipid Class Phosphorus in Normal Liver, Host Liver and Hepatoma Lipid from Rats Fed Methyl 2-Hexadecenoate[a]

Lipid class & quantity	Percentage P in lipid class from			
	Control liver	Liver, 16≡1 diet	Host liver 16≡1 diet	Hepatoma, host 16≡1 diet
Quantity PL (mg/g wet wt)	40.2 ± 3.0	34.5 ± 0.6	33.8 ± 5.7	11.1 ± 1.0
Orig.	2.3 ± 2.6	2.5 ± 1.2	2.3 ± 0.2	3.2 ± 0.2
Lyso PC	2.8 ± 1.2	6.7 ± 2.2	4.4 ± 0.4	4.2 ± 1.8
SPH	5.0 ± 0.6	5.9 ± 0.5	4.4 ± 0.4	12.6 ± 0.9
PC	48.3 ± 7.0	42.8 ± 2.3	44.7 ± 3.3	34.8 ± 0.7
PI	9.9 ± 0.7	8.4 ± 0.6	9.7 ± 2.5	9.1 ± 0.7
PS	4.6 ± 2.6	8.1 ± 2.4	4.2 ± 0.3	5.7 ± 1.9
PE	19.9 ± 2.2	17.5 ± 2.9	24.4 ± 4.5	23.7 ± 1.9
DPG & SF	7.4 ± 1.1	6.5 ± 1.1	6.3 ± 0.3	7.0 ± 1.1

Abbreviations: PL = total phospholipids; orig. = origin of thin-layer chromatogram; Lyso PC = lypophosphatidylcholines; SPH = sphingomyelins; PC = phosphatidylcholines; PI = phosphatidylinositols; PS = phosphatidylserines; PE = phosphatidylethanolamines; DPG = diphosphatidylglycerols; SF = solvent front; and 16≡1 = methyl 2-hexadecenoate.

[a]Percentages represent the mean ± standard deviation from three or four individual animals.

Fatty Acid Composition

The fatty acid compositions of the major neutral lipid classes isolated from liver and hepatoma are given in Table IV. Hexadecenoates were dramatically elevated in all three neutral lipid classes from non-tumor-bearing animals receiving the acetylenic acid diet, but only marginally elevated in host liver. Palmitate was also elevated in all three neutral lipid classes from livers of normal and host treated animals, except cholesteryl esters of non-tumor-bearing treated animals. Combined palmitate and hexadecenoate percentages of free fatty acids, triglycerides and cholesteryl esters from non-tumor-bearing treated animals represented over 75% of the total. As a result, the other major fatty acids found in these lipid classes of normal liver were reduced in the treated animals. The fatty acid composition of control liver triglycerides (Table IV) was identical to the triglyceride composition of animals fed a fat-free diet (13) while the cholesteryl ester composition was intermediate to animals fed a chow and fat-free diet (13). The percentage distribution of the fatty acids in the hepatoma lipid classes differed dramatically from liver, was virtually unaffected by the acetylenic acid, and differed very little from the compositions reported earlier that were obtained from host animals fed chow and fat-free diets (13).

The percentage distributions of fatty acids in

phosphatidylcholines, phosphatidylethanolamines and phosphatidylinositols isolated from liver and hepatoma of animals fed methyl 2-hexadecynoate are given in Table V. Palmitate percentages were elevated in all three lipid classes of treated animals relative to control liver. Hexadecenoate percentages were also elevated in all three liver lipid classes, but phosphatidylcholines showed the largest increase. Stearate and octadecenoate percentages from treated animals were much lower than control liver percentages, except for phosphatidylinositol octadecenoates. The effect of the acetylenic acid on the percentages of the major polyunsaturated fatty acids in the liver phospholipids was minimal. Percentages of stearate in hepatoma phosphatidylcholines and phosphatidylethanolamines were reduced approximately 50% relative to previously reported percentages for hepatomas from host animals maintained on chow and fat-free diets (14). The effect of methyl 2-hexadecynoate on the other fatty acids in these two hepatoma lipid classes and phosphatidylinositol was minimal relative to tumors from chow and fat-free fed host animals (14).

Positional Fatty Acid Isomers

The percentage distribution of the *cis* hexadecenoate positional isomers of the major lipid classes isolated from hepatoma and liver of animals fed methyl 2-hexadecynoate is given in Table VI. The Δ9 isomer was the predominant isomer in all three lipid classes of hepatoma and liver from all animals. The Δ6, Δ7 and Δ11 isomers generally made up the balance of the hexadecenoates in liver triglycerides and phosphatidylcholines. Liver phosphatidylethanolamines contained only the Δ6 and Δ9 hexadecenoates and the acetylenic acid had little or no effect. The Δ7 and Δ11 hexadecenoate isomers were virtually absent from the liver triglycerides of the treated animals. The distribution of positional isomers in hepatoma triglycerides and phosphatidylethanolamines was similar and both classes contained relatively high percentages of Δ12 and Δ14 isomers, which were undetectable in liver. The characterization and identification of these two unique hexadecenoate isomers in the lipids of hepatomas will appear in a separate publication.

The percentage distributions of the *cis* octadecenoate positional isomers in five lipid classes isolated from hepatoma and liver of animals fed methyl 2-hexadecynoate are given in Table VII. Unlike the hexadecenoates, only two positional isomers, the Δ9 and Δ11, predominated. The ratio of the Δ9 isomer (oleate) to the Δ11 isomer (vaccenate) in

TABLE III

Carbon Number Distribution of Triglycerides from Hepatoma and Liver of Animals Fed Methyl 2-Hexadecynoate

Hepatic tissue and diet	Carbon number percentages[a]							
	46	48	50	52	54	56	58	60
Control liver[b]	1.8 ± 0.9	13.6 ± 0.8	38.5 ± 1.8	39.3 ± 0.9	6.6 ± 0.6	0.3 ± 0.1	---	---
Normal liver, treated[c]	2.8 ± 0.3	63.6 ± 1.2	29.8 ± 0.8	3.8 ± 1.6	---	---	---	---
Host liver, treated	8.6 ± 3.9	33.3 ± 1.2	34.7 ± 6.4	16.0 ± 1.0	6.1 ± 0.5	T[d]	---	---
Hepatoma, treated host	---	5.8 ± 1.8	5.9 ± 0.8	22.7 ± 4.6	22.0 ± 2.4	20.1 ± 0.9	14.9 ± 3.9	8.7 ± 3.1

[a]Percentages represent the mean ± standard deviation of three of four animals.
[b]Control diet consisted of fat-free + 0.5% safflower oil.
[c]Treated diet consisted of control diet + 0.1% of methyl 2-hexadecynoate.
[d]T denotes detectable amounts of less than 0.5%.

TABLE IV

Fatty Acid Composition of the Neutral Lipid Classes from Hepatoma and Livers of Animals Fed Methyl 2-Hexadecynoate

Hepatic tissue and diet	Fatty acid percentages[a]							
	14:0	16:0	16:1	18:0	18:1	18:2	20:4	Others[b]
				Free fatty acids				
Control liver[c]	1.5 ± 0.4	36.8 ± 3.8	9.2 ± 1.9	10.3 ± 4.7	36.6 ± 5.5	2.5 ± 0.5	2.6 ± 1.7	---
Normal liver, treated[d]	2.5 ± 1.9	45.8 ± 1.7	29.5 ± 7.6	5.2 ± 3.2	13.0 ± 2.6	1.9 ± 0.4	1.4 ± 0.4	---
Host liver, treated	1.0 ± 0.1	48.8 ± 7.0	13.5 ± 4.4	5.2 ± 0.8	16.6 ± 2.6	7.2 ± 2.8	4.7 ± 2.0	2.2
Hepatoma, treated host	1.1 ± 0.3	23.0 ± 3.4	1.7 ± 1.3	28.3 ± 5.1	28.8 ± 3.5	4.4 ± 2.9	4.3 ± 1.7	6.1
				Triglycerides				
Control liver	1.5 ± 0.1	37.3 ± 1.6	12.7 ± 0.1	2.1 ± 0.2	44.0 ± 1.3	1.4 ± 0.2	---	---
Normal liver, treated	1.1 ± 0.1	47.3 ± 2.2	38.1 ± 2.1	0.4 ± 0.0	11.8 ± 0.3	0.5 ± 0.1	---	---
Host liver, treated	1.3 ± 0.2	54.6 ± 9.2	13.5 ± 6.4	3.1 ± 0.7	19.9 ± 2.8	5.4 ± 1.6	1.2 ± 0.4	---
Hepatoma, treated host	0.5 ± 0.1	18.0 ± 1.0	3.5 ± 0.7	11.3 ± 0.5	25.9 ± 0.7	9.4 ± 1.4	14.6 ± 0.5	16.2
				Cholesterol esters				
Control liver	1.3 ± 0.6	39.0 ± 2.0	14.9 ± 1.6	4.5 ± 0.6	31.3 ± 2.8	3.6 ± 0.4	6.1 ± 1.5	---
Normal liver, treated	3.1 ± 1.9	31.2 ± 2.0	48.0 ± 3.1	2.3 ± 0.5	10.6 ± 1.5	2.3 ± 0.8	1.1 ± 0.5	---
Host liver, treated	1.0 ± 0.2	51.8 ± 0.4	16.0 ± 7.5	3.7 ± 0.8	14.2 ± 3.5	6.6 ± 2.2	3.6 ± 1.7	---
Hepatoma, treated host	2.3 ± 0.7	8.4 ± 1.1	4.3 ± 1.1	3.9 ± 0.4	20.2 ± 0.7	7.5 ± 1.1	27.7 ± 3.7	23.7

[a]Percentages represent the mean ± standard deviation of three or four animals.

[b]The fatty acids that composed this column of the hepatoma were primarily C-20 and C-22 unsaturated fatty acids.

[c]Control diet consisted of fat-free + 0.5% safflower oil.

[d]Treated diet consisted of control diet + 0.1% of methyl 2-hexadecynoate.

TABLE V

Fatty Acid Composition of Phospholipid Classes Isolated from Hepatoma and Livers of Animals Fed Methyl 2-Hexadecynoate

Hepatic tissue and diet	Fatty acid percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
				Phosphatidylcholines			
Control liver[b]	19.8 ± 2.2	3.5 ± 0.4	21.2 ± 0.7	21.0 ± 1.0	9.0 ± 0.2	16.7 ± 1.4	2.7 ± 0.6
Normal liver, treated[c]	30.9 ± 0.4	17.2 ± 0.7	11.9 ± 0.6	10.8 ± 0.7	8.1 ± 0.4	9.9 ± 0.6	3.1 ± 0.4
Host liver, treated	33.8 ± 3.9	7.9 ± 5.4	13.1 ± 5.2	11.9 ± 1.7	10.8 ± 1.0	12.9 ± 4.2	6.6 ± 1.4
Hepatoma, treated host	29.4 ± 0.6	3.3 ± 1.1	13.6 ± 1.4	27.8 ± 2.4	10.2 ± 1.1	9.1 ± 0.5	1.0 ± 0.1
				Phoshatidylethanolamines			
Control liver	22.1 ± 5.6	2.0 ± 0.3	27.2 ± 5.7	14.5 ± 1.4	2.7 ± 0.3	22.3 ± 8.1	6.0 ± 3.1
Normal liver, treated	28.6 ± 1.1	6.2 ± 0.8	16.7 ± 1.2	8.0 ± 0.8	2.8 ± 0.4	23.5 ± 1.1	10.4 ± 1.0
Host liver, treated	29.4 ± 3.3	3.5 ± 1.9	16.8 ± 6.3	6.9 ± 1.0	3.3 ± 0.7	21.3 ± 1.1	14.8 ± 1.7
Hepatoma, treated host	9.9 ± 0.9	1.5 ± 0.6	25.5 ± 1.9	29.5 ± 2.5	8.5 ± 0.3	14.6 ± 0.5	2.4 ± 0.3
				Phosphatidylinositols			
Control liver	5.2 ± 1.3	---	51.0 ± 1.0	5.2 ± 1.9	0.5 ± 0.2	23.2 ± 1.6	1.5 ± 0.0
Normal liver, treated	15.5 ± 1.7	2.1 ± 0.7	39.3 ± 2.8	5.6 ± 2.6	1.5 ± 0.6	21.5 ± 3.0	3.5 ± 1.4
Host liver, treated	12.4 ± 4.2	1.0 ± 0.7	40.3 ± 2.2	4.7 ± 0.8	1.8 ± 0.4	29.7 ± 4.6	4.8 ± 1.4
Hepatoma, treated host	4.5 ± 0.8	---	45.9 ± 2.3	16.1 ± 3.1	3.5 ± 0.2	24.2 ± 4.4	1.0 ± 0.2

[a]Percentages represent the mean ± standard deviation of three or four animals. The difference between the sum of any row and 100% represents minor amounts of other fatty acids not given in this table.

[b]Control diet consisted of fat-free + 0.5% safflower oil.

[c]Treated diet consisted of control diet + 0.1% of methyl 2-hexadecynoate.

normal liver was dependent on the lipid class and was affected only marginally, if at all, by the acetylenic acid. These data and those in Table VIII illustrate better than previously observed (16) how octadecenoate concentrations can be changed dramatically while the ratio of oleate to vaccenate remains unchanged in normal liver phospholipid classes. All three of the neutral lipid classes from host liver exhibited the same approximate ratio of oleate to vaccenate. Host liver phospholipids contained a higher percentage of oleate than non-tumor-bearing animals. In contrast to liver, all hepatoma lipid classes exhibited the same approximate ratio of oleate to vaccenate. The increased percentage of oleate in host liver lipid classes and the lack of lipid class oleate to vaccenate ratios in the hepatoma that are characteristic of normal liver classes are in agreement with our earlier observations (16).

DISCUSSIONS

The effect of the dietary methyl 2-hexadecynoate on the non-tumor-bearing rats was dramatic. The loss of weight during the first week, the appearance of essential fatty acid symptoms and the development of fatty livers may have resulted in part or totally from causes other than the effects on lipid metabolism described in this paper. We do not know yet whether there is a direct relation between the observed effects on lipid metabolism and the observed effects on the intact animal. Because of the serious deleterious effect of this compound, all acetylenic fatty acids should be handled cautiously until it can be determined whether the observed biological effects are associated with this particular acetylenic acid isomer or acetylenic acids in general.

Efforts to identify methyl 2-hexadecynoate or a metabolite in the lipids from liver and hepatoma were unsuccessful. This is not unexpected since it has been shown that stearolic acid undergoes both β- and ω-oxidation to yield 5-decynedioic acid (17), indicating the lack of enzymatic activity in the vicinity of the triple bond. On the other hand, this point of view is inconsistent with the observed requirements for CoA derivatives of short chain ynoic acids to be effective inhibitors of fatty acid synthesis in vitro (18) and the formation of 3-decynoate thioester by microsomal fatty acyl-CoA synthetase (19). Inhibition of fatty acid elongation (To be discussed later) may involve the CoA ester of 2-hexadecynoate, but because of the proximity of the triple bond to the carboxyl group, esterification into glycerides may be

TABLE VI

Percentage Distribution of the Hexadecenoate Positional Isomers in the Three Major Lipids Classes Isolated from Hepatoma and Liver of Animals Fed Methyl 2-Hexadecynoate

Tissue and diet	Positional isomer percentages[a]					
	Δ6	Δ7	Δ9	Δ11	Δ12	Δ14
			Triglycerides			
Control liver[b]	8.4 ± 1.7	7.2 ± 0.7	82.1 ± 3.0	2.2 ± 0.8	---	---
Liver, treated[c]	5.9 ± 1.0	--[d]	93.9 ± 1.2	---	---	---
Host liver, treated	6.1 ± 3.4	---	92.5 ± 3.0	---	---	---
Hepatoma, treated host	5.9 ± 0.4	8.1 ± 2.6	69.0 ± 4.3	1.6 ± 0.4	6.9 ± 0.5	8.5 ± 1.7
			Phosphatidylcholines			
Control liver[b]	6.9 ± 3.4	2.0 ± 1.4	86.4 ± 5.1	4.9 ± 1.1	---	---
Liver, treated[c]	5.7 ± 0.9	2.6 ± 0.6	91.7 ± 1.5	---	---	---
Host liver, treated	4.1 ± 1.8	2.2 ± 0.8	93.8 ± 1.7	---	---	---
Hepatoma, treated host	4.0	9.8	61.3	5.4	11.6	8.0
			Phosphatidylethanolamines			
Control liver[b]	5.7	---	94.3	---	---	---
Liver, treated[c]	2.1	---	97.9	---	---	---
Host liver, treated	5.0	---	95.0	---	---	---
Hepatoma, treated host			Sample lost			

[a]Percentages represent the mean ± standard deviation of three or four animals. Values without standard deviation represent the analysis of a pooled sample.

[b]Control liver was obtained from animals maintained on the fat-free diet supplemented with 0.5% safflower oil.

[c]Treatment consisted of control diet plus 0.1% methyl 2-hexadecynoate.

[d]Denotes amounts of less than 0.5%.

TABLE VII

Percentage Distribution of the Octadecenoate Positional Isomers in the Various Lipid Classes Isolated from Hepatoma and Liver of Animals Fed Methyl 2-Hexadecynoate

Lipid class	Isomer	Hepatic tissue and diet[a]			
		Control liver	Normal liver treated	Host liver treated	Hepatoma treated host
FFA	Δ9	64.0	67.7	70.9	56.3
	Δ11	36.0	30.1	27.1	35.0
TG	Δ9	75.1 ± 1.3	82.9 ± 8.4	75.6 ± 8.4	62.3 ± 5.0
	Δ11	21.6 ± 2.0	13.2 ± 0.9	20.5 ± 3.4	37.0 ± 4.3
CE	Δ9	66.5	78.3	73.9	58.9
	Δ11	29.8	19.7	24.5	39.1
PC	Δ9	38.6 ± 0.5	40.6 ± 2.4	57.7 ± 7.8	60.4 ± 3.4
	Δ11	57.6 ± 1.1	58.8 ± 3.4	38.8 ± 7.0	37.5 ± 3.5
PE	Δ9	27.4	27.2	42.3	62.4
	Δ11	65.0	66.6	51.5	35.6

Abbreviations are: FFA = Free fatty acids; TG = triglycerides; CE = cholesterol esters; PC = phosphatidylcholines; and PE = phosphatidylethanolamines.

[a]Percentages represent the mean ± standard deviation of three or four animals. Values without standard deviation represent the analysis of a pooled sample. The difference between the sum of the percentages of the two isomers and 100 represents minor amounts of other isomers not given in the table.

inhibited.

The methyl 2-hexadecynoate did not appear to exhibit any antitumor activity as we had observed earlier with eicosatetraynoic acid (unpublished data). Actually, the tumor-bearing animals on the acetylenic acid diet had a better appearance than the non-tumor-bearing animals on this diet. This subjective evaluation was supported by the fact that the host animals did not contain fatty livers (Tables I and II). Host animals livers also did not exhibit the high percentage of hexadecenoates that was observed in the non-tumor-bearing animals fed the acetylenic acid (Tables IV and V). The decreased level of hexadecenoates in the host liver lipids may have resulted from reduced desaturase activity in the host animal. We have recently shown that the Δ9 desaturase activity decreases to a very low level in the host animal liver within a week after hepatoma transplantation (20). The fall in desaturase activity and the inhibition of elongation might be expected to lead to higher palmitate percentages in the host animal than the non-tumor-bearing animals fed the acetylenic acid, as observed in most lipid classes (Tables IV and V). This previously unreported protective effect exhibited by the host animal toward a compound that affects the metabolism of normal non-tumor-bearing animals adds to the mounting evidence that indicates the lipid metabolism of the host animal is affected by the tumor (20-23).

The fatty livers of the non-tumor-bearing animals fed the acetylenic acid resulted from the accumulation of all neutral lipid classes except cholesterol (Table I). Treated non-tumor-bearing animals and control animals contained 1.9 and 1.8 mg/g wet wt, respectively. These results and the higher cholesteryl ester percentages indicate total liver cholesterol concentrations of treated non-tumor-bearing animals must have been equal to or greater than control liver. This lack of an effect of methyl 2-hexadecynoate on cholesterol synthesis is in contrast to an in vitro study where alk-3-ynoyl-CoA esters and free acids were shown to inhibit cholesterol biosynthesis via irreversible inactivation of cytoplasmic acetyl-CoA acetyl CoA acetyltransferase (19). These data suggest that the position of the triple bond can play an important role on the type of effects a ynoic acid can have on a biological system. The original use of the thioester of 3-decynoate to inhibit β-hydroxy-decanoyl thioester dehydrase, a multifunctional enzyme with *trans*-2-decenoyl and *cis*-3-decenoyl isomerase activity (24), suggested that the triple bond was transformed into an allene,2,3-decadienoate, that was the active inhibitory species (25). The circumstantial evidence that suggested the interconversion of the 3-yne to a 2,3-diene by β-hydroxy dehydrase may be correct (26), but some of the reported enzymatic inhibitions

TABLE VIII

Concentration of Monoene Fatty Acids in Various Liver Lipid Classes from Non-Tumor-Bearing Animals Fed Control and Methyl 2-Hexadecynoate Diets

	Monoene concentration (mg/g wet wt)[a]					
	Control diet			Acetylenic acid diet		
Lipid class	16:1Δ9	18:1Δ9	18:1Δ11	16:1Δ9	18:1Δ9	18:1Δ11
Free fatty acids	0.05	0.14	0.08	1.67	0.56	0.25
Triglycerides	1.94	6.14	1.77	54.62	14.92	2.38
Cholesterol esters	0.08	0.13	0.06	3.15	0.60	0.15
Phosphatidylcholines	0.58	1.57	2.34	2.34	0.65	0.94
Phosphatidylethanolamines	0.15	0.32	0.75	0.36	0.13	0.32

[a]The hexadec-9-enoates were assumed to represent 90% of the hexadecenoates in free fatty acids and cholesterol esters which are not given in Table VI.

attributed to the 2,3-diene, including those reported here, may not involve the rearrangement. This conclusion is supported by the fact that rearrangement of the 2-yne used in this study would have given rise to the 2,3-diene also that would have inhibited cholesterol biosynthesis (19) which was not observed. This argument can also be used to support the position that the 2-yne does not rearrange and that the observed effects are due to the triple bond and not rearrangement to the 2,3-diene.

Although methyl 2-hexadecynoate appeared to have little effect on cholesterol biosynthesis in the liver, the effect on fatty acid metabolism was dramatic, as shown by the massive accumulation of palmitate and hexadecenoates (Tables I, III, IV and V). The accumulation of C-16 fatty acids could have arisen from increased lipogenesis, decreased oxidation, decreased elongation or a combination of these possibilities. An increased lipogenesis alone would have given rise to a fatty acid profile similar to control liver, except for decreased levels of linoleic and arachidonic acids resulting from dilution. Robinson et al. (18) have shown that 2-ynes of short chain CoA esters act as strong noncompetitive inhibitors of fatty acid biosynthesis. Although long-chain alk-2-ynoate CoA esters were not tested as inhibitors, Bloxham (19) has reported that 3-decynoic acid did not inhibit fatty acid synthesis and this acetylenic acid could be converted to the CoA ester by a microsomal preparation. These data indicate that methyl 2-hexadecynoate or its CoA ester might inhibit fatty acid synthesis, but would not be expected to stimulate lipogenesis.

A decrease in the rate of fatty acid oxidation, without a concomitant decrease in synthesis, could lead to fatty acid accumulation. However, again one would expect the fatty acid profile to be similar to control liver, but this was not observed (Tables IV and V). Bloxham (19) had suggested that the alk-3-ynoic acid might inhibit fatty acid oxidation based upon the observed inhibition of cytoplasmic acetyl-CoA-acetyl-CoA acetyltransferase, but there does not appear to be data on the effect of long-chain alk-2-ynoic acids. The present data can not rule out the possibility that fatty acid oxidation was inhibited to some extent by the acetylenic acid, but if inhibition did occur, selectivity or preferential oxidation must have also occurred.

The most likely explanation for the accumulation of C-16 fatty acids in all the lipid classes from non-tumor-bearing animals is that the acetylenic acid inhibited elongation of saturated and monoenoic fatty acids. The concomitant decreases in the percentages of stearate and octadecenoates (Tables IV and V) are consistent with this conclusion. The decreased percentages of Δ7 and Δ11 hexadecenoates in the triglycerides of the treated animals (Table VI), presumably derived from chain shortening and elongation, respectively, supports this view. One might predict, if this hypothesis were true, that the concentration of vaccenate would be reduced since this fatty acid has been shown to be derived from the elongation of hexadec-9-enoate in liver and hepatoma cells (9,27). Neutral lipids did exhibit a decrease in the percentage of vaccenate, but the percentage of this octadecenoate isomer was unchanged in the phospholipids (Table VII). If concentrations are calculated for the octadecenoates and the vaccenate precursor, hexadec-9-enoate, a more convincing picture emerges. Although the concentration of palmitoleate in a lipid class may not hold a direct

relation to the vaccenate levels in the same class, the comparison of the concentration in several classes should indicate whether a relation exists. Table VIII indicates that vaccenate concentrations were equal to five times higher than palmitoleate concentrations in control liver, while palmitoleate concentrations in the non-tumor-bearing animals fed the acetylenic acid were up to 23 times higher than vaccenate levels. Table VIII indicates that vaccenate concentrations of phospholipid classes, which showed no detectable changes in the percentages of oleate and vaccenate in Table VII, contain less than one-half the concentrations of control values while palmitoleate concentrations were two to four times higher. These data appear to illustrate strongly that elongation of saturates and monoenes was inhibited by the methyl 2-hexadecynoate in the diet.

Methyl 2-hexadecynoate may also inhibit the elongation of polyunsaturated fatty acids. Although the unchanged to marginally lower percentages of arachidonate do not suggest this, we found the accumulation of two octadecatrienoate isomers (2% of each) in only the phosphatidylcholines of the non-tumor-bearing animals fed the acetylenic acid. The position of the double bond was not determined, but one of the isomers was probably the 6,9,12 isomers resulting from the desaturation of linoleate because of the inhibited elongation which normally preceeds desaturation. One might conclude that the other triene isomer might be linoleate, but this does not appear likely since the diet did not contain a detectable amount of naturally occurring all *cis* 9,12,15 triene.

An alternate explanation for the near normal levels of arachidonate, while the elongation of saturated and monounsaturated fatty acids appears inhibited, might be that only one of the two elongation systems was inhibited by the acetylenic acid. The microsomal system which has been suggested to be responsible for the elongation of polyunsaturated fatty acids because of higher ratios of activity on these acids (28) may not be inhibited by the acetylenic acid. This possibility is supported by the fact that the microsomal system uses malonyl-CoA for elongation, the same as fatty acid synthetase (which was not affected). If the acetylenic acid inhibits only mitochondrial elongation, essentially the reverse of the β-oxidation system (28), one might also expect fatty acid oxidation to be affected, which can not be ruled out from the present study. Methyl 2-hexadecynoate or other 2-ynoic acids may prove to be valuable compounds for determining mitochondrial contributions to lipid metabolism.

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The Effect of Feeding Di-(2-ethylhexyl) Phthalate (DEHP) on the Lipid Metabolism of Laying Hens

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ABSTRACT

Di-(2-ethylhexyl)phthalate (DEHP), a commonly used plasticizer, is now seen as an environmental pollutant. DEHP has been found to inhibit lipid and sterol synthesis in rats and mice. The effects of DEHP on various aspects of lipid metabolism were examined in chickens. White Leghorn laying hens were fed either a standard laying mash control diet (C) or the control diet containing 1% DEHP (DEHP) or 1% DEHP and 5% tallow (DEHP-T) for 28 days. DEHP and DEHP-T lowered feed consumption 10% but did not significantly affect body weight. After 3 weeks on the diets, egg production was 15-20% less in DEHP-T than in C and DEHP hens. No differences were observed in egg weight, percent shell, white or yolk among the groups. DEHP and DEHP-T did not alter egg lipid or egg cholesterol concentrations. DEHP and DEHP-T lowered plasma lipid concentration about 20% and free and total cholesterol 20-30%. Liver weights increased, being 30, 34 and 39 g for C, DEHP and DEHP-T hens, respectively, after 28 days. Total liver lipid and cholesterol increased 19% and 26% in DEHP hens and 54% and 79% in DEHP-T hens when compared to controls. In contrast, the fat content of pectoralis major muscle decreased significantly in DEHP and DEHP-T hens. These results, in showing that DEHP alters plasma and tissue cholesterol but not yolk cholesterol, demonstrate again that egg cholesterol is remarkably resistant to alteration by dietary means.

INTRODUCTION

Di-(2-ethylhexyl)phthalate (DEHP), a phthalic acid ester commonly used as a plasticizer in the production of polyvinyl chloride, is now considered an environmental pollutant (1). Since the phthalic acid esters are not chemically bound to the plastics, they can migrate from the plastics to the environment (2) and into man (3) and animals (4,5). The wide distribution in the environment, and the annual production of nearly one billion pounds of phthalic acid esters in the United States (6), has led to many studies on the toxicological and biological effects.

Recent studies have shown that DEHP affects lipid metabolism in the rat, decreasing acetate incorporation into liver lipids (7) and sterols (8) in vitro. DEHP also exerts hypolipidemic effects in rats and mice (9). These studies were done to examine the effects of dietary DEHP on lipid metabolism in the hen, and more specifically, to determine whether DEHP would lower egg yolk cholesterol. Control laying hens were compared to hens fed DEHP and to a group of hens fed DEHP and tallow. Tallow was added to insure that enough lipid was available for egg production in the event DEHP exerted severe lipid lowering effects.

EXPERIMENTAL PROCEDURES

Animals and Diets

Single-comb White Leghorn pullets were obtained from a commercial hatchery at 20 wk of age and caged individually. The birds were given a 14-hr light regimen daily and a standard breeder mash ration. When the birds reached 70% egg production, they were distributed into 3 groups of 15 each on the basis of body weight. The mean initial body weights were 1654, 1684, and 1721 g for the control (C), DEHP (DEHP) and DEHP plus tallow (DEHP-T) groups, respectively.

The control group was fed ad libitum the standard laying mash for 6 wk (1 wk pre-experimental, 4 wk experimental and 1 wk post-experimental periods). During the 4 wk experimental period, the DEHP group was fed ad libitum the standard laying mash containing 1% DEHP and the DEHP-T group was fed ad libitum the standard laying mash containing 1% DEHP and 5% tallow. During the period from days 28-35, all groups were fed the standard laying mash ad libitum. The fat content of the diets was determined gravimetrically after ethyl ether extraction using a Goldfisch fat extraction apparatus.

The control diet was a standard laying mash that contained 63.2% corn meal, 19.0% soybean meal, 5.0% alfalfa meal, 2.0% meat and bone, 7.0% limestone, 2.5% dicalcium phosphate, and 1.3% salts-vitamins-minerals. The diets containing 1% DEHP or 1% DEHP plus 5% tallow were prepared just prior to the start of the experiment by adding the appropriate amount to the laying mash and mixing. The DEHP was obtained from Eastman Kodak Co., Rochester, NY, and the tallow was stabilized white tallow. The diets were stored in a 4 C room until used. The control and DEHP diets contained 16.1% protein, 3.5% calcium, and

2727 kcal/kg metabolizable energy. The diet containing DEHP and tallow had 15.2% protein, 3.3% calcium and 2943 kcal/kg metabolizable energy. The control diet, the DEHP diet and the DEHP-T diet were 90.9, 90.6 and 88.4% dry matter, respectively. The control and DEHP diets contained 5.0% fat and the DEHP-T diet contained 9.7% fat, on a wet weight basis.

Experimental Protocol

The variables studied were: feed consumption, body weight changes, egg production, egg weight, egg composition (percent shell, white and yolk), yolk fat, total yolk cholesterol, free yolk cholesterol, plasma cholesterol (free and total), liver composition, liver lipids and cholesterol.

Blood was collected from the brachial vein of 4 hens from each group on days 0, 7, 14 and 21. Brachial vein blood withdrawal from White Leghorn hens is difficult and frequently causes substantial hematomas, consequently, the birds could not be bled from that wing vein at the succeeding time period. Plasma lipid and cholesterol data thus are not from the same hens at all time periods. The difficulty in bleeding dictated an observation number of 4 for the experimental variables at 0, 7, 14 and 21 days. At 28 days, all hens were bled and 4 hens from each group were randomly selected and killed to obtain livers and pectoralis major muscle for analysis.

The following orthogonal contrasts were studied: C vs DEHP + DEHP-T, DEHP vs DEHP-T. Analysis of covariance for heterogeneity of slopes was used to examine egg production, feed consumption and body weight data (10). The rest of the data was subjected to a one-way analysis of variance based on the a priori contrasts.

Egg Yolk Analyses

Eggs collected daily were stored at 13 C and 80% humidity. Within 4 days of collection, eggs were hard boiled, weighed and percentage of shell, white and yolk determined gravimetrically. Yolk was stored at -20 C until analyzed.

Petroleum ether-ethanol was found to extract egg yolk cholesterol satisfactorily. A weighed sample of yolk (0.8 g) was homogenized in a 50 ml teflon-lined, screw-capped test tube with 18 ml petroleum ether-ethanol (2:1) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After 6 ml of distilled water were added, the tubes were shaken by hand, phases separated by centrifuging 10 min at 260 x g, and a 1.0 ml aliquot of the petroleum ether phase was transferred to a small vial for gas liquid chromatographic analysis (GLC) of free cholesterol. After evaporation of the petroleum ether, 1.0 ml of a 20% solution of TRI-SIL (mixture of hexamethyldisilazane and trimethylchlorosilane, Pierce Chemical Company, Rockford, IL) in pyridine, containing 0.5 mg cholestane as an internal standard, was added to each vial. The GLC vial was capped and heated at 75 C for 30 min to form the trimethylsilyl (TMS) derivatives. The GLC analyses of cholesterol were made on a 6 ft glass column containing 3% OV-17 coated on GAS-CHROM Q (100/120 mesh) with a Model 7620 Hewlett Packard gas chromatograph (column temp.: 250 C, He flow rate: 30 ml/min).

Percent yolk lipids were determined by transferring a 4.0 ml aliquot of the petroleum ether phase to a weighed 50 ml screw-capped test tube, then by evaporating the solvent and weighing the residue. Total yolk cholesterol was determined by adding 0.3 ml distilled water and 6.0 ml of 2.5% KOH in ethanol to the dried residue and heating at 70 C in a shaking water bath for 90 min to saponify cholesteryl esters. Cholesterol was extracted by adding 12 ml petroleum ether and 6 ml distilled water, shaking and phasing by centrifugation. A 1.0 ml aliquot of the petroleum ether phase was taken for TMS-derivatization with GLC analysis as described for free cholesterol.

Analysis of Liver, Muscle and Blood

Samples of liver, muscle and plasma were frozen, later homogenized in petroleum ether-ethanol and the lipids extracted by the procedure outlined above for egg yolk lipids. Lipids were determined gravimetrically on dried aliquots of the extracts. Total and free cholesterol also were determined as TMS derivatives by GLC analysis as described for yolk cholesterol.

RESULTS AND DISCUSSION

Data for feed consumption and body weight are presented in Figure 1 and Table I. DEHP and DEHP-T caused a decrease in intake which was observed within the first wk (Fig. 1). Feed consumption for the entire 4-wk experimental period was 10% lower, being 102 for controls, but only 93 and 91 g/hen/day for DEHP and DEHP-T hens. During the second half of the experimental period, the DEHP hens appeared to adapt to the DEHP diet and feed consumption approached control intake. The DEHP-T hens showd a greater decline in feed consumption during the last week of the experimental period but recovered somewhat during the post-experimental period. These depressions of

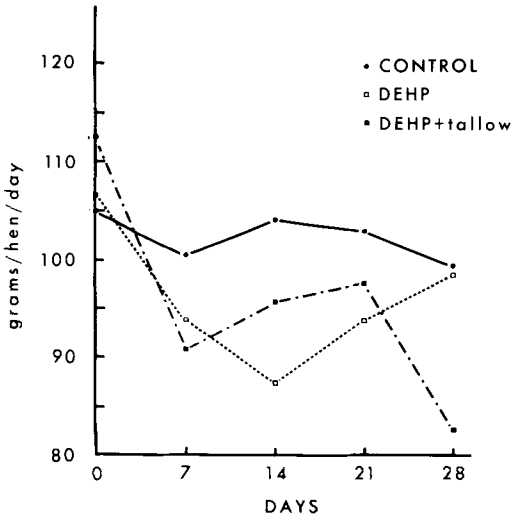

FIG. 1. Feed consumption of hens fed control, DEHP or DEHP + tallow diets for 28 days.

about 10% in feed consumption were significant at $P < .05$ (control vs DEHP and DEHP-T). No overt signs of debilitation were observed in any group. The 10% decrease in feed consumption was not reflected to the same degree in body weight changes. Consistent losses in body weight of about 2-3% were observed in the DEHP and DEHP-T hens (Table I), but these variations were not statistically significant differences.

During the third wk of feeding DEHP + tallow, egg production was 20% less than controls (Table II). Egg production for the 4-wk experimental period was 14% lower in DEHP-T, and 5% lower in DEHP, than in control hens. No differences, however, were observed in egg weight, percent shell, white or yolk among the groups (control egg data: 57.0 g egg weight, 11.0% shell, 57.2% white and 31.8% yolk). Yolk lipid and cholesterol of eggs from DEHP and DEHP-T hens were not different from controls (% lipid, 33.8; total cholesterol, 14.58 mg/g yolk; % free cholesterol, 91.0).

The effects of feeding DEHP or DEHP + tallow on plasma lipid and cholesterol concentrations, expressed as percent of control values are shown graphically in Figure 2. Because of small sample size ($n = 4$), levels during the pre-experimental period (0 wk) were higher than controls. Plasma lipids were reduced about 20% but the variations were not statistically different. Plasma total and free cholesterol were lowered about 20% relative to controls ($P < 0.01$). Free cholesterol remained about 77% of total cholesterol in C, DEHP and DEHP-T plasma.

Liver weights of the hens fed DEHP or DEHP + tallow increased (Table III) but the differences were not statistically significant. Liver composition, fat and water were not different in DEHP and DEHP-T groups compared to controls. Total liver lipid increased 19% in DEHP and 54% in DEHP-T hens ($P < 0.01$). The concentration of cholesterol and cholesteryl ester increased in the DEHP and DEHP-T livers ($P < 0.04$). Because of the accompanying increase in liver size, total cholesterol increased 29% in DEHP and 81% in DEHP-T livers.

Cholesterol and lipid were also measured in the pectoralis major muscle (Table IV). Total cholesterol and free cholesterol concentrations increased ($P < 0.05$) but fat content decreased significantly in both DEHP and DEHP-T muscle ($P < 0.05$).

Our study has demonstrated that feeding 1% of the plasticizer DEHP in the diet modified some aspects of lipid metabolism in laying hens. Body weight did not significantly change but feed consumption and egg production were lowered. The decreased feed consumption in the DEHP-T hens may have been the result of the increased caloric content of the diet. However, the caloric content of the DEHP feed was similar to the

TABLE I

Body Weight Changes of White Leghorn Hens Fed DEHP

Treatment	Initial body weight[a] (g ± SE)	Change from initial body weight (g) Days of experiment 7	14	21	28	35
Control	1654 ± 30	+18	-3	+1	+7	+33
DEHP	1684 ± 26	-99	-25	-21	-39	-25
DEHP-T	1721 ± 24	+6	-30	-56	-85	-60

[a]Values are means ± SEM for 15 White Leghorn hens in each group.

TABLE II

Effect of DEHP and Tallow Feeding on Egg Production[a]

Treatment	% Egg production during week: 1	2	3	4	5	% Egg production 0-28 days
Control	80	75	80	81	75	79
DEHP	74	69	70	77	81	73
DEHP-T	79	72	60	61	66	68
C vs DEHP and DEHP-T	NS	NS	$<.01$	$<.01$	NS	
DEHP vs DEHP-T	NS	NS	$<.01$	$<.01$	NS	
Standard error of the mean	4	4	3	3	4	

[a]Egg production was calculated weekly on a percentage basis, 100% being 1 egg/hen/day; each group consisted of 15 hens.

control diet and a depression in feed consumption was still apparent. The decreased egg production in both groups may have resulted either from their decreased feed consumption or from a physiological effect of DEHP. Liver weights increased but this trend was not statistically significant. In contrast, rats fed DEHP usually show a marked hepatomegaly (7, 11-14).

In our study, plasma lipids and cholesterol showed a 20% decrease after feeding 1% DEHP for 1 wk, effects similar to those observed in rats and mice (9,11,14). However, much greater effects were observed on lipid and cholesterol levels in the chicken liver: total lipid and total cholesterol content increased about 70%. Plasma cholesterol decreased in DEHP-fed rats

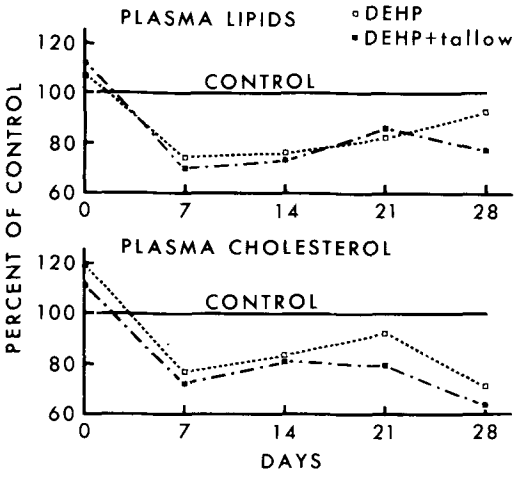

FIG. 2. Plasma lipids and cholesterol of hens fed control, DEHP or DEHP + tallow diets for 28 days. The overall means (± SE) of control plasma concentrations were: total lipids 2382 ± 144 mg/100 ml; cholesterol 127 ± 6 mg/100 ml. n at 0, 7, 14, 21 days: 4; n at day 28:15.

and rabbits without an accompanying increase in liver cholesterol concentration (11,15). Studies in rats, however, generally show increases in the total lipid and cholesterol content of the liver of about this magnitude when placed on a whole organ basis (11,14,16). Thus, our results confirm that DEHP affects lipid metabolism in an avian species in a manner similar to that reported for mammalian species.

The mechanism by which cholesterol lowers plasma lipids and cholesterol has not been elucidated. The presence of desmosterol was not observed in the plasma, liver or muscle samples of the hens in this experiment, indicating the cholesterol and lipid reduction did not take place by inhibition of the Δ^{24}-sterol reductase step in the conversion of desmosterol to cholesterol (17).

Several hypotheses have been proposed for the hypolipidemic mechanism of action of DEHP: (a) increased lipid oxidation caused by DEHP- induced peroxisome proliferation; (b) decreased lipid biosynthesis; (c) inhibition of energy-linked reactions, and (d) feedback control of cholesterol on its own biosynthesis.

Peroxisome Proliferation

Liver peroxisome proliferation is characteristic of hypolipidemic compounds and has been demonstrated for many unrelated lipid lowering agents (18-22). Several catabolic, lipid-related enzymes have been shown to be associated with liver peroxisomes, including catalase (23), carnitine acetyl transferase (24) and recently, the β-oxidative enzymes (25). An increase in liver catalase and carnitine acetyl transferase activity was found when DEHP was fed to rodents (9,13,26). In addition, liver β-oxidation increased significantly in rats administered DEHP for 1 wk (26). Therefore, increased activities of catabolic lipid enzymes associated with liver peroxisomes could be

TABLE III

Effect of Feeding DEHP or DEHP + Tallow in Hepatic Parameters of the Hen[a]

	Liver weight g	% of body weight	% H_2O	% Fat	Total lipid g	Cholesterol mg/g DW	Cholesteryl ester mg/g DW	Total cholesterol mg	Total cholesteryl ester mg
Control	29.6	1.84	69.6	17.3	5.1	7.8	0.8	68.5	6.7
DEHP	34.1	2.07	70.4	17.8	6.1	8.7	1.4	88.1	13.5
DEHP + Tallow	38.8	2.29	68.7	20.3	8.1	10.2	2.0	123.7	24.5
Standard error of the mean	2.8	0.2	1.1	1.8	1.2	0.6	0.2	9.8	2.8
C vs DEHP and DEHP-T	NS	NS	NS	NS	NS	<.04	<.02	<.01	<.01
DEHP vs DEHP-T	NS	NS	NS	NS	NS	NS	NS	<.01	<.01

[a]Values are the means of 4 hens.

responsible for DEHP's plasma lipid lowering effects.

Decreased Lipid Synthesis

A consistent decrease in labeled acetate and mevalonic acid incorporation into various lipid fractions was found in both in vitro and in vivo experiments with rats (7-8,11,27-28). Decreased incorporation of label into squalene and sterols was observed and significant reductions in fatty acyl lipids were also noted, implying inhibition of an enzyme common to both groups. A decrease in acetate activation has been suggested as a possibility (11), since that enzyme would be required for de novo synthesis of cholesterol and fatty acyl lipids. A reduction in acetate incorporation before an effect upon mevalonic acid incorporation into rat liver squalene and sterols suggested a possible site for DEHP's inhibitory effect on liver cholesterol biosynthesis would be at the enzyme 3-hydroxy-3-methyl-glutaryl CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (11).

Inhibition of Energy Linked Reactions

A reduction in liver electron transport enzyme activities and inhibition of succinic dehydrogenase and adenosine triphosphatase in heart, liver, kidney, testes and ovaries of DEHP-treated rats have been found (15). In addition, an inhibition of liver mitochondria respiration and uncoupling of oxidative phosphorylation by phthalate esters (29-30) has led to a conclusion that the suppression of energy-linked reactions may be a generalized DEHP effect on cholesterol content (16).

Feedback Control

The accumulation of liver cholesterol could inhibit 3-hydroxy-3-methyl-glutaryl CoA reductase, reducing liver synthesis of cholesterol (31). Accumulation of liver cholesterol on a total liver basis was found in hens fed DEHP. It is possible this resulted in decreased liver cholesterol biosynthesis which reduced circulating cholesterol.

Egg lipids and cholesterol were unaffected in spite of a 20% reduction in plasma cholesterol, demonstrating that egg cholesterol is resistant to alteration by dietary agents (32,33). This may reflect the removal of a negative feedback inhibition upon ovarian cholesterol biosynthesis. As plasma cholesterol falls, the feedback inhibition on ovarian cholesterol biosynthesis may be removed, resulting in normal levels of yolk cholesterol, even though plasma cholesterol has been depressed. Signifi-

TABLE IV

Effect of Feeding DEHP or DEHP + Tallow on Cholesterol and Lipid Content of the Pectoralis Major Muscle[a]

Group	Total cholesterol mg/100 g WW	Free cholesterol mg/100 g WW	Lipid %
Control	60.9	55.9	4.3
DEHP	67.1	57.8	3.6
DEHP + Tallow	73.7	65.7	1.6
Standard error of the mean	3.4	2.7	0.6
C vs DEHP and DEHP-T	$P<0.05$	NS	$P<0.05$
DEHP vs DEHP-T	NS	$P<0.05$	$P<0.05$

[a]Values are the means of 4 hens.

cant in vitro ovarian cholesterol synthesis has been reported (34). However, in vivo experiments indicate that little, if any, ovarian synthesis occurs under normal conditions (35).

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Vitamin A Induced Hypertriglyceridemia in Cholesterol-Fed Rats[1]

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ABSTRACT

The effects of level and feeding frequency of retinoic acid (OIC) or retinyl acetate (YL) on the accumulation of lipids in the serum and liver of rats were investigated. Male Sprague-Dawley rats were fed ad libitum 1% cholesterol diets with or without supplemental OIC or YL. Vitamin A-fed groups included (per g of dry diet): 105 μg of OIC or 113 μg YL daily for 28 days, 735 μg OIC or 791 μg YL once each week for 28 days; and 735 or 105 μg OIC on day 1 or 105 μg OIC daily for the week experiment. Daily feeding of OIC or YL increased serum triglyceride concentrations as compared to controls. Several days after removal of OIC or 1 week after removal of supplemental YL from the rat diets, serum triglyceride concentrations returned to basal levels. Cholesterol feeding elevated serum cholesterol as well as hepatic cholesterol, total lipids and vitamin A concentrations. Daily OIC feeding depressed serum and hepatic cholesterol concentrations. These results show that daily supplement of vitamin A increased serum triglycerides and reduced serum and hepatic cholesterol concentrations. Serum and liver alterations were dependent on continued feeding.

INTRODUCTION

Studies have demonstrated antihypercholesterolemic effects of vitamin A and β-carotene administered to cholesterol-fed rats (1,2). Erdman et al. (3-4) reported various active forms of vitamin A, especially retinoic acid (OIC), not only reduced cholesterol synthesis but also stimulated glyceride synthesis in post-mitochondrial supernatant fractions of rat liver homogenates.

The purpose of these experiments was to determine the effects of OIC feeding upon triglyceride and cholesterol accumulation in the serum and liver of cholesterol-fed rats.

METHODS

Experiment 1

Table I shows the experimental design and dietary constituents for the first experiment. Forty male Sprague-Dawley rats weighing 120-160 g were weight-averaged into 5 groups. Groups B-E received a 1% cholesterol conditioning diet for 8 days whereas group A received the control diet for this period. After conditioning, the groups underwent a 7-day experimental period (groups B-E continued to receive the 1% cholesterol control diet). For the first day, groups C and D received 735 and 105 μg of OIC per g of diet (dry basis), respectively, whereas group E was fed 105 μg OIC per g diet for the duration of the experimental period. All diets contained a basal level of 6.0 μg retinyl acetate (YL) per g diet. This represents approximately 10 times the RDA for the rat (5).

All diets were gelled by heating distilled water to 100 C, removing the heat and vigorously mixing the water with an equal weight of dry ingredients (Table I). The order of addition of ingredients was: agar dispersed in sucrose, casein, corn starch, cottonseed oil (with or without dispersed cholesterol) and mineral mix. Crystalline cholesterol was first dissolved in absolute ether. This mixture was added to warmed cottonseed oil, and the ether was dissipated by bubbling nitrogen through the stirred oil (see reference 6). After sufficient cooling, the vitamin mix was added (with or without OIC. OIC (compliments of Hoffmann-LaRoche Inc., Nutley, NJ) was added to diets in crystalline form. Diets were evenly blended, poured into plastic tubs with air-tight covers, gelled and refrigerated until use. The efficacy of gel diet use in lipid studies and the procedure for dispersing cholesterol in oil were reported previously (6).

In order to minimize light destruction of vitamin A, the opaque gel diets were fed daily at 1800 hr – the beginning of the dark cycle. Diet and water were provided ad libitum. Blood was obtained periodically during the experiments and prior to sacrifice via heart puncture. All heart punctures were performed between the hours of 0600 and 0900, thus the experimental periods were in half-day multiples. The rats were not fasted, therefore the time of the most recent intake of food in relation to the time of bleeding cannot be established. One-half of the animals from the experimental group were killed after 6½ and 27½ days for experiments 1 and 2, respectively. The remaining animals were killed after 7½ or 28½ days. The final results have been labeled as day 7 or

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day 28 results, whereas serum data prior to the kill day have been referred to as half-day data. At the end of the feeding period, livers were removed, rinsed, blotted dry and frozen at -20 C until analysis. Whole blood was centrifuged and serum was decanted and frozen.

Experiment 2

This experimental design was similar to experiment 1 except there was no preconditioning period. Rats were fed for 4 weeks, repeating the 7-day design of experiment 1 four times. An additional form of vitamin A, YL (Sigma Chemical Co., St. Louis, MO), was fed at 113 or 791 μg/g of diet. These fortification levels correspond on a molar basis to dietary OIC levels. For this experiment, weanling rats were fed the various gel diets according to the design described in the lower portion of Table I.

ANALYTICAL METHODS

Total serum cholesterol was determined colorimetrically with a stable Liebermann-Burchard cholesterol reagent (Hycel Inc.,

TABLE I

Experimental Design and Dietary Makeup for Experiments 1 and 2

Experiment 1 (7 days)

Diet composition[a] and vitamin A administration	Dietary groups[b]				
	Control -chol	Control +chol	735 OIC +chol (day 1)	105 OIC +chol (day 1)	105 OIC +chol (days 1-7)
	A	B	C	D	E
1% cholesterol[c,d] addition	---	+	+	+	+
Vitamin A[f] supplementation	---	---	735 μg OIC[g] /g diet on day 1	105 μg OIC[g] /g diet on day 1	105 μg OIC /g diet for 7 days

Experiment 2 (28 days)

Diet composition[a] and vitamin A administration	Dietary groups[b]					
	Control -chol	Control +chol	735 OIC +chol (days, 1, 7,14,21)	791 YL +chol (days 1, 7,14,21)	105 OIC +chol (days 1-28)	113 YL +chol (days 1--28)
	A	B	C	D	E	F
1% cholesterol[d,e] addition	---	+	+	+	+	+
Vitamin A[f] supplementation	---	---	735 μg OIC[h] /g diet on days 1,7,14,21	791 μg YL[h] /g diet on days 1,7,14,21	105 μg OIC /g diet for 28 days	113 μg YL /g diet for 28 days

[a]Basal diet consisted of 43.0% starch, 21.7% sucrose, 22.0% casein, 6.0% cottonseed oil, 4.0% mineral mix, 1% vitamin mix and 2.0% agar. For make-up of vitamin mix and mineral mix see ref. 6. Agar was bacteriological grade by Gibco, Grand Island, NY. All other ingredients were supplied by Teklad, Madison, WI.

[b]OIC = retinoic acid, YL = retinyl acetate.

[c]Cholesterol diet fed for 8-day preconditioning period and 7-day experimental period.

[d]Cholesterol added to basal diet at the expense of 0.5% starch and 0.5% cottonseed oil.

[e]No cholesterol preconditioning period was used in experiment 2.

[f]The basal diet vitamin mix contains 6.0 μg retinyl acetate/g diet.

[g]On days 2-7 these groups received control diet B.

[h]On days 2-6, 8-13, 15-20, 22-28 these groups received control diet B.

TABLE II

Final Mean Weight, Serum and Hepatic Lipids for Rats Fed Different Levels of Retinoic Acid (Experiment 1; 7 Days)

Analyses	Control[a] (-chol) A	Control (+chol) B	735 OIC (+chol) (day 1) C	105 OIC (+chol) (day 1) D	105 OIC (+chol) (days 1-7) E
Determinations/group	8	8	7	8	8
Weight gain, g[b,c]	82.0 ± 5.4	78.2 ± 8.7	76.7 ± 9.3	84.4 ± 10.8	81.1 ± 5.6
Hepatic weight, g	12.2 ± 0.7	13.2 ± 1.3	13.2 ± 1.3	13.5 ± 1.9	13.9 ± 1.6
Hepatic lipids, mg/g	32.23 ± 3.13[a]	58.5 ± 6.2[b,c]	62.8 ± 6.0[b]	56.7 ± 7.26[c]	60.1 ± 4.0[b,c]
Hepatic cholesterol mg/g	2.32 ± 0.39[a]	5.9 ± 1.1[b]	5.6 ± 0.9[b,c]	5.0 ± 0.7[c,d]	4.5 ± 1.0[d]
Hepatic phospholipids mg/g	10.2 ± 1.05	10.4 ± 1.2	10.5 ± 1.0	10.7 ± 1.7	10.8 ± 1.1
Hepatic vitamin A μg/g	7.14 ± 2.7[a]	30.4 ± 9.1[b]	28.0 ± 6.4[b]	33.1 ± 15.4[b]	33.8 ± 15.4[b]
Serum triglycerides mg/100 ml	175.1 ± 50.4[a]	220.4 ± 69.9[b]	214.8 ± 38.2[a,b]	218.0 ± 50.0[b]	308.7 ± 33.8[c]
Serum cholesterol mg/100 ml	98.2 ± 12.9[a]	114.8 ± 12.9[b]	104.2 ± 13.1[a,b]	102.7 ± 12.0[a]	115.2 ± 20.7[b]
Serum vitamin A μg/100 ml	23.9 ± 7.1	20.4 ± 8.4	22.4 ± 3.0	23.1 ± 4.1	16.2 ± 6.8

[a]See Table I for full explanation of experimental groups.

[b]Values in the same line not sharing a common superscript letter are significantly different ($P < 0.05$).; Data points represent means ± S.D.

[c]Weight gain data represents total gains in conditioning period and experimental period.

Houston, TX). Serum triglycerides were analyzed by triglyceride reagent kit (Sigma Chemical Co., St. Louis, MO). Hepatic lipids were extracted with chloroform/methanol (2:1) using a modification of the procedure outlined by Folch et al. (7). Hepatic phospholipid and cholesterol were assayed by the methods described by Bartlett (8) and Zlatkis et al. (9), respectively. Hepatic and serum vitamin A analyses were accomplished spectrophotofluorometrically following the procedure of Thompson et al. (10).

RESULTS

Experiment 1

The results from the first experiment are found in Table II. No significant differences in mean weight gains during the total (conditioning-plus-experimental) feeding period were observed. Analysis of weight gains during the conditioning or the experimental period (not shown) also revealed no difference between groups. Cholesterol feeding significantly ($P < 0.05$) increased total hepatic lipids, hepatic cholesterol and hepatic vitamin A but had no effect upon hepatic phospholipid concentrations. Feeding of 105 μg of OIC per g diet (groups D and E), but not 735 μg (group C) resulted in reduced hepatic cholesterol concentrations compared to the cholesterol-fed control group B ($P < 0.05$).

Figure 1 shows the serum triglyceride levels of animals from groups B through E obtained at days 1½, 2½, 4½, 5½, and day 7. Elevated triglyceride concentrations were noted after 1½ days in both 105 μg OIC-fed groups (D and E) ($P < 0.05$), compared to the 1% cholesterol control. At day 2½ and 4½ only group E, which was still receiving OIC, demonstrated elevated triglyceride concentrations ($P < 0.05$). No significant differences between groups were noted after 5½ days. The final data again showed that daily administration of OIC elevated serum triglyceride concentrations ($P < 0.01$). The final triglyceride levels of all groups are found in Table II. It should be noted that cholesterol feeding also elevated serum triglycerides.

Serum from intermediary heart punctures revealed no differences in cholesterol concentrations between groups (not shown). Final serum cholesterols were slightly elevated in cholesterol-fed groups and only group D exhibited significantly lower serum cholesterol concentrations compared to group B, the cholesterol-fed control. No significant differences in final serum vitamin A were found; however, serum vitamin A for the animals that received

OIC for the entire experimental period (group E) was lower (N.S.) than all other groups.

Experiment 2

The results of the 28-day study are found in Table III. OIC feeding resulted in lower weight gains of animals, probably caused by reduced feed intake in the 105 OIC (group E) and by reduced feed efficiency in the 735 OIC (group C) ($P < 0.05$). Some group mean liver weights were larger than the non-cholesterol-fed control, group A. When liver weights were put on a percent of body weight basis (not shown), no differences between groups were noted. Cholesterol feeding resulted in increased total hepatic lipids and hepatic cholesterol concentrations compared to the non-cholesterol control (group A). Feeding 105 or 735 ng of OIC (groups C and E) resulted in significantly depressed hepatic cholesterol concentrations compared to the cholesterol-fed control (group B). YL feeding increased hepatic vitamin A; weekly feeding resulted in greater liver accumulations of the vitamin than daily feeding.

Serum analysis revealed that final serum triglyceride concentrations were elevated by daily feeding of YL ($P < 0.05$) or OIC ($P < 0.01$). Weekly doses of either form of the vitamin had no significant effect on serum lipids after 28 days of feeding. Serum cholesterol was slightly elevated when cholesterol was fed, and was reduced to non-cholesterol-fed control levels (group A) with the daily OIC treatment (group E).

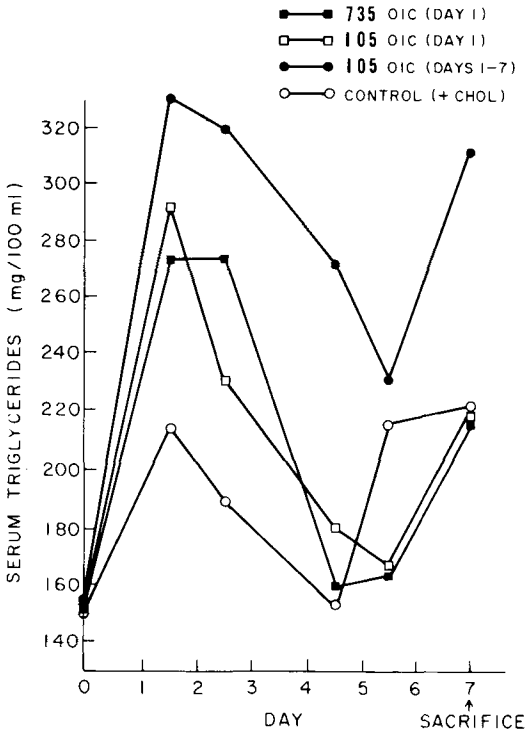

FIG. 1. Effect of feeding frequency and level of retinoic acid (OIC) upon serum triglycerides, exp. 1. Each data point on day 1½, 2½, 4½ or 5½ represents the average serum level from blood obtained by heart puncture from three animals. Data points at day 7 represent the average of 7 or 8 animals sacrificed on day 6½ or 7½. Symbols: ■—■ = 735 μg OIC/g diet provided on day one only; □—□ = 105 μg OIC/g diet provided on day one only; ●—● = 105 μg OIC/g diet provided throughout the experimental period; ○—○ = 1% cholesterol control diet.

DISCUSSION

The most notable observation in this set of experiments was the elevation of serum triglyceride concentrations resulting from the OIC and YL dietary regimens. From Figure 1, it is clear that 1½ days after feeding 105 μg OIC/g diet, serum triglyceride concentrations increased. A few days after cessation of OIC feeding, the serum triglyceride concentration returned to the control non-fasted level. The short duration of elevated serum triglycerides after removal of OIC from diets corresponds closely to the purported metabolic active life of OIC of about 2 days (11). The higher dose level (735 μg of OIC did not cause any greater increase in triglyceride concentrations than was seen with the lower level. The daily OIC feeding regimen maintained elevated serum triglycerides except at 5½ days after the start of the experiment.

No explanation can be offered for the variability of the serum triglycerides over the week. A similar weekly rhythmic elevation and decline of triglycerides was noted in serum from heart punctures (not shown) obtained periodically during the 4-week experiment.

The hypertriglyceridemic effect of vitamin A was substantiated in experiment 2, as two analogs (OIC or YL) of the vitamin were fed over a longer period. After 28 days of feeding, serum triglyceride concentrations were elevated in both groups receiving daily administration of either form of vitamin A (Groups E and F; Table III). Those animals who received vitamin A administration once weekly failed to show hypertriglyceridemia at the end of 28 days. It should be noted that even though YL is metabolically active longer than OIC (11,12), YL did not maintain elevated serum triglycerides except when administered on a daily basis. Excess dietary YL is taken up by the liver, stored and released to tissues as required. The results from this study suggest the stored vitamin A may have no functional role in the ele-

TABLE III

Final Mean Weight, Serum and Hepatic Lipids for Rats Fed Different Levels of Retinyl Acetate or Retinoic Acid (Experiment 2; 28 Days)

Analyses	Control -chol[a]	Control +chol	735 OIC +chol (day 1,7,14, 28)	791 YL +chol (day 1,7,14 28)	105 OIC +chol (days 1-28)	113 YL +chol (days 1-28)
	A	B	C	D	E	F
Determinations/group	8	8	8	6	8	8
Weight gain, g[b]	169.2 ± 11.7[a,b]	170.3 ± 11.6[a,b]	156.6 ± 7.7[c]	167.6 ± 10.9[b]	155.4 ± 8.6[c]	177.4 ± 7.4[a]
Feed intake, g day dry diet	12.8 ± 0.69[a,b]	13.1 ± 0.86[a]	12.4 ± 0.50[b]	12.9 ± 0.43[a,b]	11.78 ± 1.38[c]	13.41 ± 0.32[a]
Feed efficiency g gained/g intake	0.472 ± .019[a]	0.464 ± .012[a,b]	0.446 ± .014[b]	0.464 ± .020[a,b]	0.475 ± .060[a]	0.471 ± .017[a]
Hepatic weight, g	10.3 ± 0.8[a]	11.8 ± 1.0[b,c]	10.4 ± 0.9[a]	10.8 ± 1.6[a,b]	10.3 ± 0.6[a]	11.9 ± 1.1[c]
Hepatic lipids, mg/g	36.7 ± 1.6[a]	77.1 ± 17.0[b]	67.8 ± 6.8[b]	69.1 ± 11.8[b]	72.5 ± 17.2[b]	79.5 ± 11.1[b]
Hepatic cholesterol mg/g	2.07 ± 0.93[a]	7.31 ± 1.13[b]	5.13 ± 0.92[c]	6.54 ± 0.84[b,d]	6.16 ± 1.17[c,d]	7.58 ± 1.50[b]
Hepatic vitamin A μg/g liver	2.8 ± 1.2[a]	22.0 ± 10.2[a]	24.0 ± 15.1[a]	447.0 ± 114.3[b]	6.4 ± 3.3[a]	316.3 ± 126.9[c]
Serum triglycerides mg/100 ml	148.7 ± 53.0[a]	157.0 ± 40.8[a]	148.7 ± 46.1[a]	141.8 ± 40.6[a]	230.2 ± 36.0[b]	206.5 ± 62.2[b]
Serum cholesterol mg/100 ml	97.9 ± 8.9[a]	111.7 ± 12.6[b]	106.3 ± 14.6[a,b]	113.9 ± 17.3[b]	96.9 ± 7.4[a]	114.9 ± 13.2[b]

[a]See Table I for full explanation of experimental groups.

[b]Values in the same line not sharing a common superscript letter are significant different ($P < 0.05$). Data points represent means ± S.D.

vation of serum triglyceride concentrations.

The underlying mechanism of increased serum triglyceride concentrations is unknown. Since the liver functions as a major site for synthesis of serum triglycerides, vitamin A may stimulate hepatic triglyceride synthesis and/or release. Recent studies from this laboratory using fasted rats fed with or without OIC (13) have indicated that elevation of plasma triglycerides by OIC is not chylomicron-linked. Increased serum triglyceride concentrations may reflect decreased breakdown of VLDL triglyceride and/or reduced uptake by tissues. We have substantial evidence that the adrenal gland was not mediating vitamin A's lipogenic effect (14). This work also suggested fatty acids were not being mobilized from adipose tissue to generate triglycerides.

OIC feeding significantly reduced group mean weight gains in the 4-week, but not in the 1-week experiment. Although growth was reduced by less than 10%, the reduction suggests a mild toxicity occurred when feeding rats approximately 1300 μg OIC per day for 28 days. A possible relationship between vitamin A toxicity and the genesis of elevated lipids may exist but remains unanswered at this time. OIC feeding of 105 μg/g diet for 7 days or 26 μg/g diet for 28 days did not cause weight reduction in non-cholesterol-fed rats (14). The oral LD_{50} of all-*trans* OIC for mice is 1.1 g per kg body weight (15). This dose level is over 125 times the level fed to rats in the present study. Food intake data from this study indicates rats fed a 105 μg/g diet consumed from 1200-1600-ng OIC per day. In this study, YL feeding at 113 μg/g diet for 28 days had no adverse effect on growth.

Further studies of vitamin A and other retinoid-induced hypertriglyceridemia is necessary considering the potential applications of retinoids as antitumorogenic agents (16). Recent anticarcinogenic feeding studies with rats have successfully used levels of the 13-*cis* isomer that were 3 times greater than the levels of the all-*trans* OIC used in the present study (17-18).

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The Free Radical Oxidation of Polyunsaturated Lecithins

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ABSTRACT

Two unsymmetric polyunsaturated lecithins were allowed to air oxidize and the primary products of autoxidation were isolated and characterized. 1-Palmitic-2-linoleic-phosphatidylcholine undergoes significant oxidation after 16 hr at room temperature under air. A new phospholipid product may be isolated by reverse phase high pressure liquid chromatography (HPLC) and this HPLC fraction is shown to be made up of lipid hydroperoxides formed by free radical oxidation of the homoconjugated diene of the linoleate component of the lecithin. 1-Stearic-2-arachidonic-phosphatidylcholine undergoes a similar oxidation with the arachidonate polyunsaturated functionality being oxidized. The structure of the oxidation products was established by reduction of hydroperoxide with triphenylphosphine, snake venom hydrolysis of the C-2 ester, and HPLC analysis of the resulting hydroxy fatty acids or their methyl esters.

INTRODUCTION

The oxidation of membrane phospholipid has been suggested to be the primary chemical reaction in a variety of pathological events. For example, membrane damage induced by radiation (1) and carbon tetrachloride or ethanol poisoning (2-4) has been proposed to be the result of phospholipid destruction by molecular oxygen. In fact, a theory of aging has been proposed that rests, in part, on the free radical oxidation of membrane lipid (5).

In light of the potential importance of phospholipid oxidation products, it seems remarkable that little effort has been made to purify and chemically characterize these species. Oxidation of polyunsaturated phospholipid leads to conjugated diene as detected in the ultraviolet and also to peroxide products (6) as indicated by titration. However, no primary phospholipid oxidation products have as yet been purified and the standard methods of phospholipid purification (silica gel column and thin-layer chromatography) would appear to offer little hope for isolation of these primary oxidation products.

Recently, high pressure liquid chromatography (HPLC) has been shown to be a powerful tool for use in the purification of fatty acid oxidation products. For example, the lipid hydroperoxide products formed from lipoxygenase reactions of arachidonic acid (7,8) have been prepared by air oxidation of the fatty acid and purified by HPLC (9,10). Further, the first isolable products in prostaglandin biosynthesis, PGG_2 and PGH_2, (11,12) have recently been purified by HPLC (13). PGG_2 and PGH_2 are both peroxide products of limited stability as are the lipoxygenase products 12-hydroperoxy and 5-hydroperoxy arachidonic acid. The fact that these compounds may be purified by HPLC suggested to us that HPLC might also be used in the study of phospholipid oxidation.

We recently reported (14) that different lecithin molecular species could be separated by reverse phase HPLC and the primary components of egg lecithin could also be purified by this technique. Further, it was noted that after storage at low temperature for several months, 1-palmitic-2-linoleic-phosphatidylcholine was converted to a new product. This new product was readily separated from the unreacted lecithin by reverse phase HPLC and we suspected it to be an oxidation product of 1-palmitic-2-linoleic-phosphatidylcholine. We report here on the isolation and characterization of lecithin air oxidation products. HPLC may be used to purify the primary lecithin oxidation products and we note that these primary oxidation products are lecithin hydroperoxides.

MATERIALS AND METHODS

Fatty acids were obtained from NuChek Prep (Elysian, MN) and used without further purification. Egg lecithin was obtained from Sigma Biochemical Co. (St. Louis, MO).

Lecithin Synthesis

1-Palmitic-2-linoleic-phosphatidylcholine (1P, 2L-PC) was prepared by the method reported by Gupta et al. (15). Thus, egg lecithin was converted to glycerophosphorylcholine which was then diacylated to give dipalmitic phosphatidylcholine (di P-PC). Snake venom hydrolysis followed by reacylation gave 1P, 2L-PC. The product was characterized by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and was pure by thin-layer chromatography (TLC) and HPLC. 1-Stearic-2-arachidonic phosphatidylcholine was prepared by a similar approach (15).

Autoxidation of 1P, 2L-PC

The 1P, 2L-PC solution was evaporated to dryness under vacuum so that the lecithin formed a thin layer on the inside of a round bottom flask. The flask was then exposed to a stream of dry air, and the reaction was monitored by reverse phase TLC (RP-TLC) and RP-HPLC. After 16 hr the primary product was isolated by RP-HPLC and analyzed.

TLC

Normal phase TLC was carried out on silica gel 60F-254 precoated plates (Merck), using $CHCl_3$-MeOH-H_2O (70:25:4, v/v) as the eluent. This gives a good separation of lecithins from lysolecithin (16), but an incomplete resolution of 1P, 2L-PC and its primary oxidation product. The oxidized product moved somewhat slower than the starting material.

Reverse phase TLC (RP-HPLC), precoated silanized silica gel 60F-254 (Merck) was carried out using MeOH-NH_4OH-0.6% $CHCl_3$ (7:2:1, v/v) as the mobile phase. This method gave an excellent separation of the lecithin and its primary oxidation products, and was therefore useful in monitoring the oxidation in an early stage. However, in a later stage, other oxidation products gave rise to very large spots interfering with both the starting material and the primary oxidation products. Phospholipids were visualized with molybdenum blue spray (zinzadre reagent) (16) and peroxides were detected with the KI/starch assay (17).

HPLC

A μ Bondapak C-18 column (Waters Assoc., Milford, MA) was used with CH_3OH-H_2O-$CHCl_3$ (solvent proportions described in figure captions) as the eluent (14). The flow rate was 3.0 ml/min; detection was by refractive index or UV (240 nm). For preparative purposes, large amounts (20 mg in 200 μl solvent) of the oxidation mixture could be separated per injection. Hydroxy fatty acid methyl esters were separated by normal phase HPLC using a Waters Associates 10 μ Porasil column (7.8 mm ID x 30 cm) and .5% EtOH in hexane as the eluent. The flow rate was 4.0 ml/min, detection was by UV at 235 nm. Free hydroxy fatty acids were analyzed using acetic acid-isopropanol-hexane (1:16:983, v/v) as solvent and the same 10 μ column as was used for the hydroxy fatty acid methyl ester analysis.

Analysis of Lecithin Autoxidation Products

Fractions collected by reverse-phase HPLC were concentrated in vacuo at ambient temperature until only water remained. The residue was freeze dried or evaporated to dryness after adding CH_2Cl_2 and MeOH, to obtain azeotropic water removal. Samples of the isolated product were reduced with triphenyl phosphine (10). The reduced lecithin was then specifically hydrolyzed at the C_2 position with phospholipase A_2 (Sigma, *Crotalus adamanteus* snake venom) to isolate the 18:2 hydroxy fatty acids (16). This was done at ambient temperature to keep decomposition or isomerization of the product to a minimum. The hydroxy fatty acid mixture was methylated with diazomethane (10) to obtain the methyl esters, which were separated by normal phase chromatography (18). For purposes of efficiency in routine analyses, the methylation step was omitted and the hydroxy fatty acids were analyzed by HPLC. The order in which the isomers were eluted was the same as for the methyl esters. This was found by methylating each hydroxy fatty acid separately and resubjecting it to chromatography. The isolated hydroxy fatty acid methyl esters were characterized by GC-MS after hydrogenation over PtO_2 and trimethyl silylation with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (19). Characteristic fragmentation patterns (19) indicated that the hydroxyl groups were formed at the C_9 and C_{13} positions. GC-MS was carried out with a 3 ft OV-1 column at a temperature ranging from 200-230 C over a 10 min period. The silylated saturated 9- and 13-hydroxy methyl esters had retention times of 9.3 and 9.7 min, respectively.

RESULTS

1-Palmitic-2-linoleic-phosphatidylcholine reacts readily with oxygen when neat films, aqueous emulsions, or solutions (t-butanol, benzene solvent) of the lecithin are exposed to oxygen. The oxidation of thin films is particularly rapid at room temperature but dilute solutions are considerably more stable to air than the neat lecithin.

The reaction of the polyunsaturated lecithins with oxygen is inhibited by butylated hydroxy toluene (BHT), a known free radical inhibitor, and the oxidation is thus apparently a free radical chain process (see Discussion).

After exposure of neat 1-palmitic-2-linoleic-phosphatidylcholine to oxygen at 25 C for 16 hr, significant oxidation of the lecithin had occurred. Figure 1 shows the HPLC chromatogram obtained from such an oxidation, with two new fractions, **1** and **2,** being observed in addition to the starting lecithin, **3.** Oxidation of

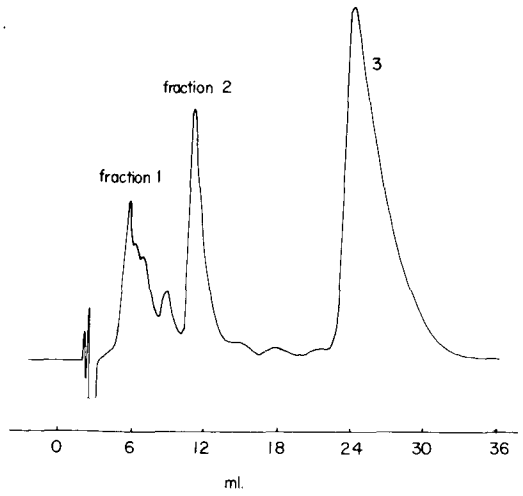


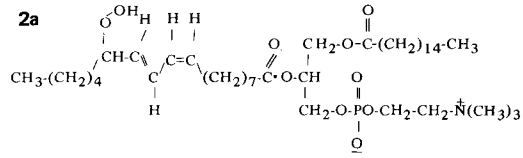
FIG. 1. HPLC chromatogram of product mixture obtained from a 16 hr oxidation of 1-palmitic-2-linoleic phosphatidylcholine. Solvent, CH_3OH-H_2O-$CHCl_3$ (100:10:10, v/v); 10 μ C-18 reverse phase column; detection by refractive index.

275 mg of 1-palmitic-2-linoleic-phosphatidylcholine for 16 hr (about 20% theoretical oxygen consumption) led to 32 mg fraction **2** and 6.6 mg fraction **1** as isolated by HPLC. By following an oxidation over time with HPLC, it was determined that fraction **2** was the first formed lecithin oxidation product. When fraction **2** was isolated, allowed to stand at room temperature and then rechromatographed, significant amounts of fraction **1** had been formed from **2**. Thus, fraction **1** appears to be a secondary oxidation product formed by decomposition of the compound(s) making up **2**. Fraction **2** is apparently identical to the previously reported (14) contaminant of 1-palmitic-2-linoleic phosphatidylcholine formed upon storage at -20 C for several months.

Fraction **2** showed a maximum in the ultraviolet at 230-235 nm, indicating the presence of a conjugated diene. Further, it was shown to be a peroxide by the potassium iodide/starch test. NMR spectroscopy of fraction **2** showed that the bisallylic protons (2.8 δ) -CH=CH-CH_2-CH=CH- present at C-11 of linoleic acid had disappeared and the vinyl region had been broadened, an observation consistent with the formation of a conjugated diene. We thus tentatively concluded that fraction **2** was a diene hydroperoxide, or a mixture of various diene hydroperoxides, like **2a**.

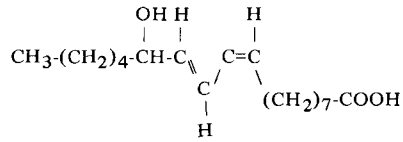
Triphenylphosphine reduction of **2** produced a new product, **4**, that was no longer

2a



peroxide positive, but still contained a conjugated diene as determined by its UV spectrum. Snake venom hydrolysis of **4** followed by HPLC analysis of the resulting free fatty acid led to the HPLC chromatogram displayed in Figure 2. Four major products were observed in the chromatogram, and these four products have been assigned the structures **5a-5d. 5a,** the first hydroxy fatty acid eluted, contains a 13-hydroxyl group with 9-*cis*,11-*trans* diene stereochemistry. **5b** is a 13-hydroxy-9-*trans*,11-*trans* compound and **5c** and **5d** are the 9-hydroxy *trans,cis* and *trans,trans* dienes,

5a



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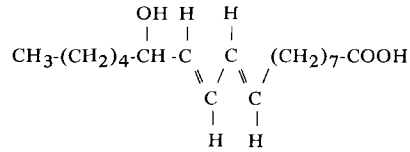

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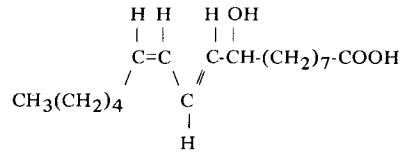
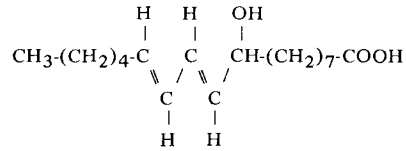


5d



respectively. These structural assignments were based on: (a) spectral comparison of the products obtained from methylation of **5a-5d** with the hydroxy linoleate methyl esters previously prepared and chromatographed by Chan and Levett (18) and (b) hydrogenation of the methyl esters (vide supra) followed by silylation and GC/MS analysis to indicate the position of oxygen substitution on the carbon chain. It should be noted that the diene stereochemistry of the hydroxy linoleate methyl esters was previously established by infrared and ultraviolet spectroscopy and we have

confirmed these stereochemical assignments. The ratio of products formed in the autoxidation was 13, *cis,trans,* 18%; 13, *trans,trans,* 34%; 9, *trans,cis,* 14%; and 9, *trans,trans,* 34%.

1-Stearic-2-arachidonic-phosphatidylcholine, **6**, is even more reactive toward oxidation than was the palmitic-linoleic lecithin. After only 5 hr, significant oxidation of **6** had occurred and the HPLC trace shown in Figure 3 was obtained, As can be seen from the chromatogram, several products are formed from the lecithin. Separation of the various oxidation products by HPLC followed by: (a) triphenylphosphine reduction, (b) snake venom hydrolysis, and (c) HPLC analysis of the hydroxy arachidonate methyl esters (10) led to the conclusion that the fraction eluting from the HPLC column at 28 ml (fraction 7) contained the primary products of **6** oxidation, the arachidonic ester hydroperoxides. The product distribution of hydroxy arachidonate methyl esters obtained by the sequence outlined here was very similar to the distribution obtained from air oxidation of arachidonic acid methyl ester (9). The air oxidation of **6** thus follows a course similar to oxidation of 1-palmitic-2-linoleic-phosphatidylcholine. That is, conjugated diene hydroperoxides are formed first and these primary compounds decompose to give a complex mixture of products.

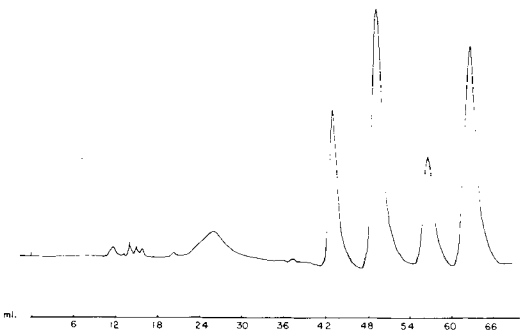


FIG. 2. HPLC chromatogram of hydroxylinoleate methyl esters obtained from 1-palmitic-2-linoleic phosphatidylcholine oxidation. Solvent ethanol-hexane (5:995, v/v); detection by UV at 235 nm; 10 μ silica column, 7.8 mm x 30 cm.

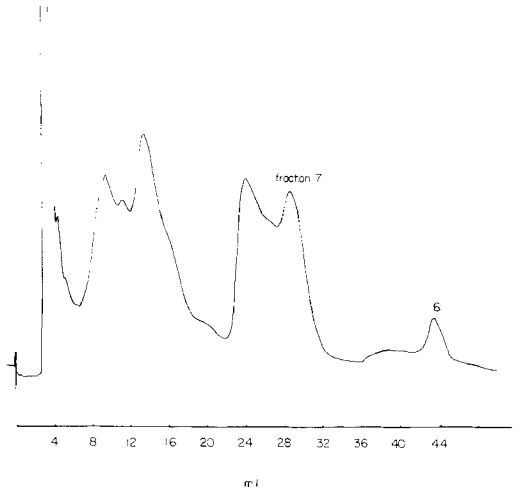


FIG. 3. HPLC chromatogram of product mixture obtained from a 5 hr oxidation of 1-stearic-2-arachidonic phosphatidylcholine. Solvent, CH_3OH-H_2O-$CHCl_3$ (100:12:10, v/v); 10 μ C-18 reverse-phase column; detection by UV at 240 nm.

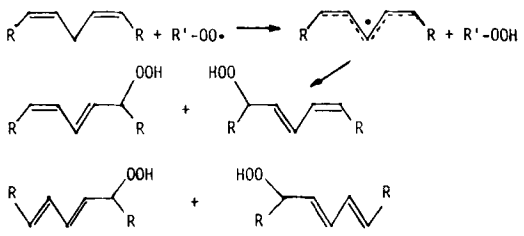
DISCUSSION

Polyunsaturated fatty acids and esters are particularly prone to undergo air oxidation by a free radical chain mechanism. Autoxidation has been investigated in detail (20) and the generally accepted mechanism involves the two propagation steps (I) and (II). The overall rate of autoxidation is related directly to kp,

$$R\text{-}O\text{-}O\bullet + R'\text{-}H \xrightarrow{kp} R\text{-}OOH + R'\bullet \quad (I)$$

$$R'\bullet + O_2 \rightarrow R'\text{-}OO\bullet \quad (II)$$

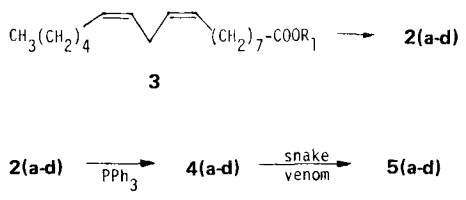
the rate constant of hydrogen atom abstraction by a peroxy radical. This rate constant, kp, is dramatically dependent on the structure of R'-H and the homoconjugated diene present in linoleic as well as other polyunsaturated fatty acids and esters is a particularly good hydrogen atom donor (21). Abstraction of the bisallylic hydrogen of the homoconjugated diene leads to a free radical that is stabilized by delocalization over five carbon atoms and reaction of this radical with molecular oxygen followed by hydrogen atom abstraction leads to the hydroperoxides shown (Scheme I).



SCHEME I.

Polyunsaturated lecithins are noted for their instability in air. 1-Palmitic-2-linoleic-phosphatidylcholine, **3**, readily undergoes air oxidation and our evidence indicates that a mixture of

four hydroperoxides, **2(a-d)**similar to those shown in Scheme 1 is formed. Reduction of the hydroperoxides with triphenylphosphine to give the alcohols **4(a-d)** is followed by snake venom hydrolysis, a method known to specifically remove the fatty acid ester from position 2 of the glycerol moiety. The four hydroxy fatty acids formed by this sequence are shown here to be identical to the alcohols formed from autoxidation of free linoleic acid or its methyl ester (18). (Scheme II).



R_1 = 1-palmitoyl glycerophosphatidylcholine

SCHEME II.

The autoxidation of polyunsaturated lecithins thus appears to follow a course similar to the autoxidation of the free fatty acids or esters, with conjugated diene hydroperoxides being the primary products formed in the free radical chain process. Both the hydroperoxides **2(a-d)** and the alcohols **4(a-d)** are not separated from the starting material by normal phase chromatography, whereas RP-HPLC or RP-TLC readily separate these compounds from the starting lecithin.

The procedures reported here make available lecithin hydroperoxides for potential biological study. Further, the methods outlined here provide a framework for the study of the oxidation of more complex mixtures of lecithins such as those found in biological membranes. In this regard, we are currently investigating the oxidation of egg lecithin aqueous emulsions and our preliminary observations suggest that the approach may well provide detailed chemical information about the oxidation of model membrane systems.

ACKNOWLEDGMENTS

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During the course of this study, we became aware of similar work on soybean phospholipids carried out by Dr. C.G. Crawford et al. of USDA-Peoria Labs. We thank Dr. Crawford for helpful discussions.

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A Lipid Mobilizing Factor in Serum of Tumor-Bearing Mice

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ABSTRACT

There is considerable evidence that the growing tumor requires a source of unsaturated fatty acids, but the nature of this source and the mechanism of mobilizing the fatty acids from it are obscure. These experiments make use of AKR mice with implanted adipose tissue labeled with 1-^{14}C linoleic acid. With this experimental animal, it has been found that: (a) in the normal, fed mouse, fat is mobilized slowly and appears largely as respiratory CO_2, following oxidation, (b) in the normal, fasted mouse, fat is mobilized rapidly and appears largely as respiratory CO_2; (c) in the tumor-bearing, fed mouse, fat is mobilized rapidly and appears largely in the tumor; and (d) the serum from tumor-bearing mice, when injected into normal mice, produces an immediate massive fat mobilization that does not respond to feeding, whereas the serum from normal, fed mice does not. It is concluded that a mobilizing factor of unknown nature is present in the serum of tumor-bearing AKR mice.

INTRODUCTION

Many observations have indicated a growing tumor is able to derive the fatty acids necessary for membrane formation from the host tissues. For example, Smedley-Maclean and coworkers (1,2) reported implanted Walker tumors caused a decrease in polyunsaturated fatty acids in surrounding tissues. Mead and Decker (unpublished results) found that chronically fat-deficient mice died with acute fat deficiency symptoms following transplantation of an Ehrlich tumor. The source of the fatty acids was not clear from these and similar experiments. Studies specifically designed to answer this question have not been helpful, possibly because of the heterogeneity of the tumors used and the different reactions in different experimental animals. For example, Spector and Brenneman (3) reported little intraperitoneally injected glucose radioactivity appeared in the tumor lipid of Ehrlich ascites tumor-bearing mice, and Baker and his associates (4,5) have reported an impairment of lipid transport in these mice and have indicated the liver is not the direct source of the hypertriglyceridemia seen.

On the other hand, it seems evident that in mammals, at least, the major source of rapidly available fatty acid must be adipose tissue and many observations of tumor-bearing animals and patients have revealed a picture of fat depletion with advanced malignancies.

In preliminary experiments in this laboratory, it was found that in AKR mice with advanced lymphomas, injection or oral administration of [1-^{14}C]linoleic acid resulted in relatively increased incorporation into liver and thymus phospholipids and decreased incorporation into triglycerides as compared to controls (6). The hypothesis advanced at the time was that, in tumor-bearing mice, an increased transport of fatty acid to the tumor and increased incorporation into tumor cell membranes was occurring. Observations of this sort have been made previously (7).

Clearly, there is a need for a definitive study to identify the source of tumor lipid, the means of transport to the tumor and the mechanism by which the lipid transfer is induced. These experiments were designed to answer those questions, at least for one animal and type of tumor.

MATERIALS AND METHODS

Experimental Animals

Both male and female AKR mice were used. This strain has an almost 100% mortality from thymic lymphoma between the ages of 6 and 14 months with a peak incidence at 9-10 months (8). 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 (9). Animals were housed in groups of 6-7 and fed laboratory mouse chow ad libitum.

Animal Procedures

[1-^{14}C]Linoleic acid was administered orally to a healthy AKR mouse and after 4 hr the mouse was sacrificed and the resulting labeled retroperitoneal and suprapelvic adipose tissue was removed and divided into pieces of about 0.1 g (100,000 cpm). These were transplanted into different locations of the peritoneal cavity of age-matched lymphoma-bearing and control AKR mice under light ether anesthesia (10). Vascularization of the transplanted adipose tissue occurred in about 2 days, as shown microscopically, and these mice were

used for the experiments described in the following text. In all, 8 lymphoma-bearing mice and an equal number of normal controls were used. In some cases, mice bearing radioactive grafts were used for several experiments and thus served as their own controls. Animals with both spontaneous and accelerated lymphoma were used as recipients of labeled adipose tissue grafts. The serum for lipid mobilization experiments was taken from 2 to 3-month-old mice with accelerated lymphoma and from 1-month-old healthy controls.

For collection of respiratory CO_2, special plexiglass cages holding a single mouse were used. (These cages were generously furnished by Dr. M. Ookhtens of the Tumor Lipid Laboratory, Wadsworth Veterans Administration Hospital, Los Angles, CA) (11). The cages had one air inlet allowing air to enter from the rear and one outlet directly in front of each mouse to permit collection of expired CO_2. Dimensions of the cages were such that the mice could not turn around throughout the period of breath collection. Air was swept at a rate of 500 ml/min/cage (dead space swept at least 10 times per min) and bubbled through 40 ml of hydroxide of hyamine 10-x (Rohm and Haas) solution. The 0.032 M solution was prepared in absolute ethanol containing phenolphthalein as an indicator. Each CO_2 collection funnel was supplied with 40 ml of this solution. Disappearance of the pink color of the solution indicated the completion of the titration, i.e., the saturation of the hyamine by expired CO_2. Four separatory funnels were arranged to collect CO_2 from a pair of mice simultaneously. Aliquots of hyamine solution were collected after the pink color of the hyamine solution disappeared and were used for ^{14}C assay.

Liquid Scintillation Counting

Aliquots (5 ml) of the hyamine solution were taken after disappearance of the pink color and were mixed with 10 ml of Dimilume-30 in glass counting vials and kept in the dark at 4 C overnight. Appropriate blanks and experimental samples were counted on a Beckman LS 8100 liquid scintillation spectrometer.

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.

Radio Gas Liquid Chromatography

Methyl esters of component fatty acids were prepared from the separated lipids by methanolysis with 4% HCl in methanol for 1 hr at 80 C. The methyl esters were extracted with pentane and were analyzed for mass and radioactivity on the Packard, Model 824, gas chromatograph with gas proportional counter, Model 894, fitted with a 6 ft x 4 mm glass column with a liquid phase of Apolar 10 C.

RESULTS

Findings after Transplantation of Adipose Tissue

Figure 1 shows transplanted adipose tissue in a lymphoma-bearing mouse. There are intact blood vessels containing red blood cells. New vascularization has taken place between the peritoneum and transplanted adipose tissue. In this mouse, the transplanted adipose tissue had decreased in size during 3 days. About 80% of the radioactivity from the transplanted tissue was found incorporated in the various tumor tissues – liver, thymus, spleen, mesenteric

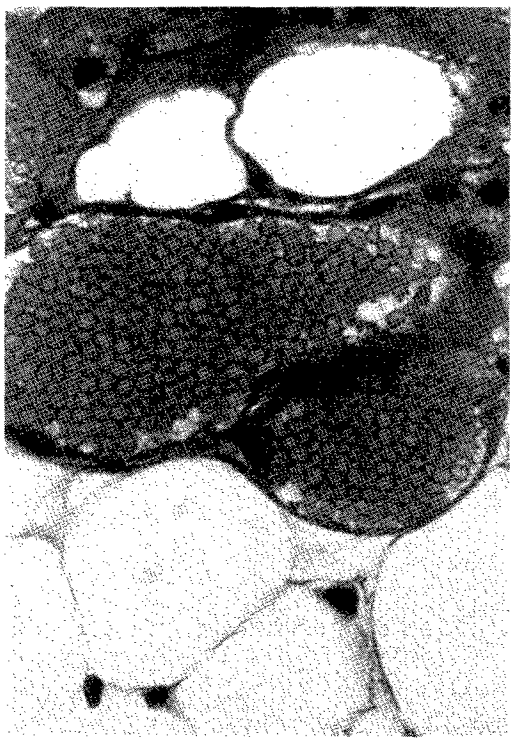

FIG. 1. Transplanted adipose tissue from a lymphoma-bearing mouse. (Data shown in Table I). Hematoxylin and eosin are used for staining; figure is magnified x 400. The graft in this lymphoma mouse survived as well as that in control mice.

lymph nodes, inguinal lymph nodes, and cervical lymph nodes, for example.

In advanced lymphoma cases, thymus, lymph nodes and spleen cells were largely replaced by lymphoma cells, and in the liver extensive invasion by lymphoma cells was seen, particularly around the blood vessels. In control mice, the grafts in general had more extensive vascularity and better survival than those in the lymphoma-bearing mice. The control grafts did not decrease noticeably in size and little radioactivity was found in tissues other than the transplanted adipose tissue.

Analysis of Tissue Lipids

As shown in Tables I and II, after sufficient time for vascularization of the transplanted tissue, significant amounts of radioactivity were found in all tumor-containing tissues – liver, thymus, spleen and lymph nodes. Considerable variability occurred in the incorporation of radioactivity into the tissues of these mice because of several factors such as stage of lymphoma and vascularization of graft. Only small amounts of radioactivity were found in the liver and thymus of control mice. The most extensive mobilization was found in those animals in which the best vascularization occurred, as seen microscopically. In the tumor tissues, most of the radioactivity was found in the phospholipid fraction, as reported previously, following [1-^{14}C]linoleic acid administration (5). Radio gas chromatography showed the fatty acid radioactivity pattern of the phospholipid from tumor tissue was 85% linoleic acid and 15% arachidonic acid (Fig. 2).

Fasting Experiments

Since lipid mobilization also is caused by fasting, a comparison was made of the transfer of radioactivity induced by fasting or by the growing tumor. In a sense, this had already been done since it had been found that, in the normal mice, radioactivity from adipose tissue was not extensively transferred to other tissues, whereas in the tumor-bearing mice much of the radioactivity was found in the tumor or in the tissues, such as the liver, which were extensively invaded by tumor. These findings take on additional significance since it was found that the tumor-bearing mice ate approximately the same amounts as the controls (3-4 g/day).

For the fasting study, mice with the radioactive grafted adipose tissue were placed in the metabolism cages, radioactive CO_2 was collected and the radioactivity per mmol CO_2 was measured. In the cases of both fed control and lymphoma-bearing mice, a low level (less than 100 cpm/mmol CO_2) was produced throughout the period. Prolonged fasting in the normal mouse with radioactive adipose tissue graft, however, produced an increase in expired $^{14}CO_2$ with a peak value of 1700 cpm/mmol (which corresponds to about 3000 cpm/hr). This was confirmed by collecting CO_2 for 1 hr with a more concentrated hyamine solution. When the fasting was interrupted by feeding a minimal amount of diet (0.5-2 g), the radioactive CO_2 fell to baseline values precipitously and then slowly rose as fasting was resumed.

TABLE I-A

Radioactivity Incorporated into Various Tumor Tissues in Lymphoma-Bearing Mouse

	Radioactivity in total lipid (cpm)	Radioactivity in phospholipids (cpm)	Radioactivity in neutral lipid (cpm)
Liver	57340	41930 (80%)	10810 (20%)
Thymus	7620	6610 (89%)	820 (11%)
Spleen	11830	10870 (97%)	290 (3%)
Inguinal and cervical lymph nodes	8280	5017 (67%)	2460 (33%)
Mesenteric lymph node	2150		

TABLE I-B

Distribution of Radioactivity in Neutral Lipids of Thymus and Liver of Lymphoma-Bearing Mouse

	FFA cpm	FFA/TL%	Chol. cpm	Chol./TL%	TG cpm	TG/TL%	Chol. E cpm	Chol. E/TL%
Liver NL	50	0.1%	438	0.8%	7770	14%	730	1.3%
Thymus NL	7	0.1%	74	1.0%	395	5%	200	2.6%

TABLE II

Radioactivity Incorporated into Tissues of Lymphoma-Bearing and Control Mice

Mice		Sex	Days after transplantation	Radioactivity in liver TL (cpm)	Radioactivity in thymus TL (cpm)	Radioactivity in mesenteric lymph node TL (cpm)
Lymphoma	(1)	F	6	6650	473	1195
	(2)	F	4	9330	1660	910
	(3)	M	3	57340	7620	2150
	(4)	F	5	23970	2810	9850
	(5)	M	3	3920	733	
	(6)	F	4	8030	680	
	(7)[a]	M	4	3635	530	
	(8)[a]	F	3	5610	540	
Control	(1)	F	5	560	44	
	(2)	M	6	380	12	
	(3)	M	3	840	33	
	(4)	F	4	385	260	
	(5)	M	5	665		
	(6)	F	4	200		
	(7)	M	3	380		
	(8)[b]	F	4	665		

M = Male; F = Female.

[a]Lymphoma (7) and (8) are spontaneous lymphoma mice. (Lymphoma [7] = 9 months old, Lymphoma [8] = 7 months old).

[b]Control (8) is a 1-year-old healthy mouse. The rest of the lymphoma mice are virus accelerated (2-3 months old). The rest of the control mice are age-matched (2-3 months old).

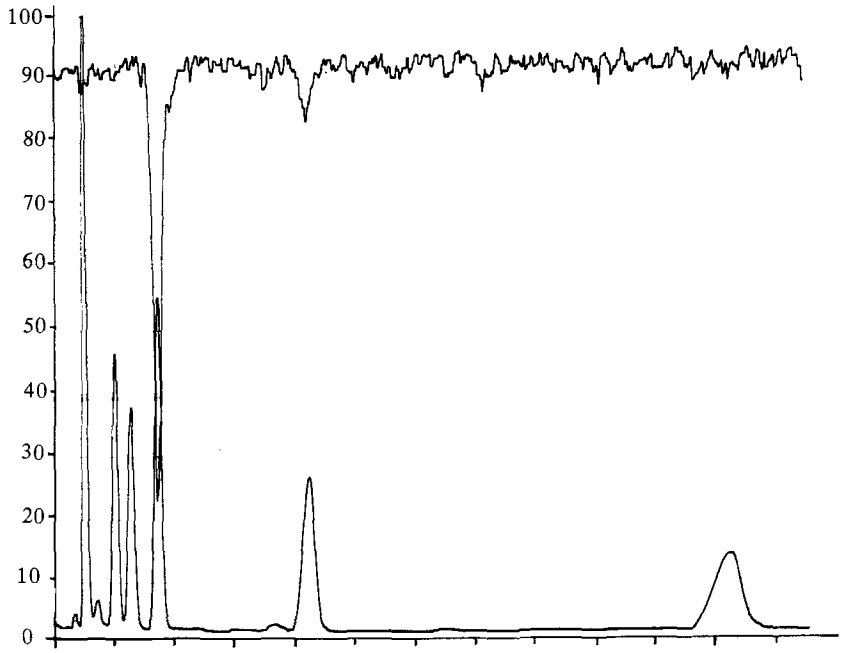

FIG. 2. Liver phospholipid fatty acid pattern from lymphoma-bearing mouse. Mass peaks (lower curves) are from a standard mixture. Radioactivity is present only in 18:2 and 20:4.

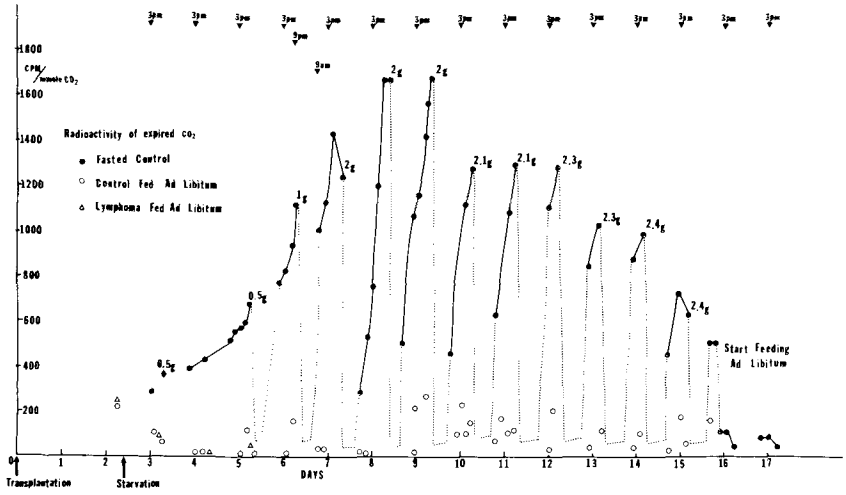

FIG. 3. Radioactivity of expired CO_2. The abscissa shows days after transplantation. Fasting started 2½ days after transplantation of labelled adipose tissue. The ( )g shows the amount of food given to the fasted mouse.

This process could be repeated continuously, each cycle bringing about an increase (fasting) or decrease (feeding) of radioactivity. In this manner, the radioactivity in the transplanted adipose could be, in a sense, titrated until all the radioactivity of the adipose tissue had appeared as $^{14}CO_2$ and this was confirmed, after sacrifice, since negligible radioactivity remained in the graft. One such series is shown in Figure 3. It should be emphasized that the conditions in this experiment are different from those that follow in that the former represents severe starvation and the latter mild fasting.

Test for Mobilizing Factor

Since, by the method outlined above, each mouse could be its own control, an experiment was initiated in which a normal mouse bearing a radioactive adipose tissue graft was tested for quantitative response to feeding or fasting. As usual, feeding 0.5 g food brought about a precipitous decline in radioactive CO_2 emission to very low values within 45 min. As fasting progressed, the radioactivity slowly increased, reaching 150 cpm/mmol in 3 hr (Fig. 4). A more gradual increase was seen following 1.0 or 1.5 g food; in the latter case, 150 cpm/mmol was reached in 6-7 hr (Fig. 4).

From the information gained in this experiment, the following studies were conducted after ¼-day of fasting – allowing liver glycogen to be largely depleted then giving 2 g food and additional food at appropriate times – in such a way that the effect of fasting was not a factor in the studies.

Two hr following the feeding of 2.0 g chow, when the radioactive CO_2 was at a low point, 0.3 ml of serum from a mouse with advanced lymphoma was injected by tail vein. There was an immediate dramatic increase in expired radioactivity (to 200 cpm/mmol) and a further increase to 300 cpm/mmol in 5 hr (Fig. 5). At 9 hr, 0.5 g food was given but did not result in a decrease of radioactivity. Injection of 0.3 ml serum from a normal, fed control mouse did not result in any change under the identical experimental conditions (Fig. 5).

This experiment was repeated using a mouse in which, following feeding, the expired CO_2 had an activity of 5.6 cpm/mmol. Injection of 0.3 ml of serum from a lymphoma-bearing mouse caused an increase to 120 cpm/mmol.

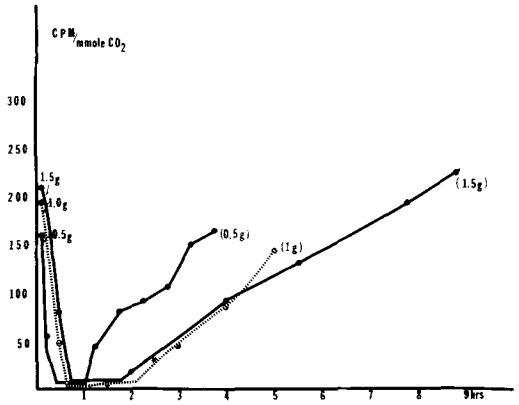

FIG. 4. Appearance of respiratory $^{14}CO_2$ in mouse with radioactive adipose tissue graft with time and treatment (see Results). (g) Indicates the amount of food given at time 0.

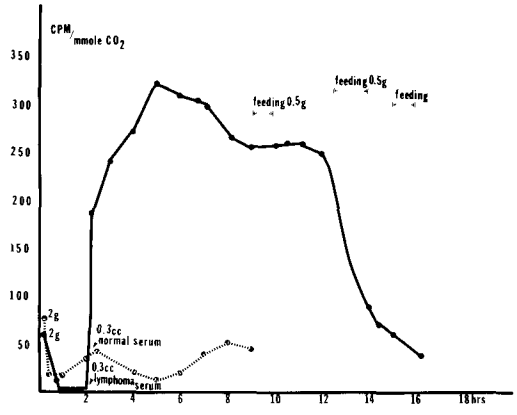

FIG. 5. Appearance of respiratory $^{14}CO_2$ in mouse with radioactive graft with time and treatment (see Results). ( )g Indicates the amount of food given at time 0 or the indicated time periods. Arrows show times of injections.

An extract of thymus from a lymphoma-bearing mouse caused an increase to 134 cpm/mmol, whereas an extract of pooled thymus glands from two control mice had no effect. Also, injection of conditioned medium from growth phase culture of an AKR SL3 lymphoma cell line (13) caused an increase to 134 cpm/mmol. No increase was observed with serum from a control mouse or control culture medium. (RPMI 1640 with 10% fetal calf serum).

DISCUSSION

The use of mice bearing transplanted radioactive adipose tissue permits the rapid and sensitive assessment of the state of mobilization of adipose tissue. It is evident that the graft behaved qualitatively similarly to the endogenous adipose tissue since under conditions of release of radioactivity from the graft, the endogenous adipose tissue decreased in size. A check on the measurement of $^{14}CO_2$ appearance was obtained after sacrifice, by measuring the radioactivity of all tissues. This indicated that in the fed normal mouse, mobilization is slow and much of the released fatty acid is oxidized. In the fasted normal mouse, mobilization is rapid and, again, most of the released fatty acid is oxidized. In contrast, in the fed, tumor-bearing mouse (these animals did not survive fasting), most of the fatty acid was mobilized but, in this case, it appeared in the tissues.

As discussed above, there is ample evidence that growing tumors require a source of unsaturated fatty acids for formation of membrane phospholipids. There is also a great deal of indirect evidence implicating fatty acids as growth promoting agents in carcinogenesis (14-15). In the same light, indirect evidence has supported the idea that mobilizing factors are produced by the growing tumor: the unsaturated fatty acids are made available for its growth. The above experiments furnish direct evidence that there is a potent mobilization inducing factor in the serum of tumor-bearing mice but not in that of controls. That it is different from the mobilization inducing factors (e.g., epinephrine) produced in fasting is evident, since feeding has little or no effect on the tumor serum induced mobilization, whereas it inhibits that produced by fasting. Since the factor is also present in tumor extract and in culture medium from lymphoma cell culture, it is evidently not produced indirectly from some other tissue but is a direct product of the tumor.

Future experiments will include attempts to isolate and identify the factor or factors. It is anticipated that these results will require time but will be immeasurably aided by the technique described in this paper.

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Inhibition of Mitochondrial Fatty Acid Elongation by Antibodies to 3-Ketoacyl-CoA Thiolase

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ABSTRACT

Antibodies to pig heart 3-ketoacyl-CoA thiolase inhibited almost completely and in a parallel fashion thiolase and the acetyl-CoA-dependent fatty acid elongation system present in an acetone powder extract of pig heart mitochondria. This finding leads to the conclusion that mitochondrial fatty acid elongation occurs by reversal of fatty acid oxidation. Several lines of evidence point to the thiolase-catalyzed condensation reaction as the rate-limiting step in the formation of elongated products. However, the accumulation of hydroxy acids suggests the enoyl-CoA reductase activity is limiting in the synthesis of saturated fatty acids.

INTRODUCTION

Fatty acid synthesis in mitochondria occurs by an acetyl-CoA-dependent elongation of fatty acids (1). A number of details for this metabolic pathway as well as its physiological function have not yet been elucidated. Specifically, the nature of the enzymes involved in this process has not been established although it has been suggested the elongation occurs by reversal of β-oxidation, except that the last step is catalyzed by a distince NADH- or NADPH-dependent enoyl-CoA reductase (1,2). This suggestion is supported by the observed elongation of acyl-CoA primers in the presence of a mixture of purified enzymes consisting of 3-ketoacyl-CoA thiolase (EC 2.3.1.16), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), enoyl-CoA hydratase (EC 4.2.1.17) and eoyl-CoA reductase (EC 1.3.1.8) (2). However, other reports represent evidence that the fatty acid elongation activity behaves like a single protein with a molecular weight of maximally 135,000; these reports do not agree with the proposed involvement of β-oxidation enzymes in this process (3,4).

In order to determine whether mitochondrial fatty acid synthesis occurs by reversal of fatty acid oxidation, we studied the effect of antibodies to 3-ketoacyl-CoA thiolase on the elongation activity. Also, we attempted to identify the rate-limiting step in this pathway.

EXPERIMENTAL PROCEDURES

Materials

Acetyl-CoA, CoASH, octanoyl-CoA, NAD^+ and NADH were obtained from P-L Biochemicals, Inc. (Milwaukee, WI). [1-^{14}C]-Acetyl-CoA and [1,3-^{14}C]malonyl-CoA were purchased from New England Nuclear, Inc. (Boston, MA). The following substrates were synthesized according to established procedures: Acetoacetyl-CoA (5), 3-ketodecanoyl-CoA (2), $\Delta^{2,3}$-decenoyl-CoA (6). *d,l*-3-Hydroxydecanoyl-CoA was prepared from *d,l*-3-hydroxydecanoic acid and CoA by the Goldman and Vagelos mixed anhydride method (7). The concentrations of all acyl-CoA compounds except for that of 3-ketodecanoyl-CoA were determined by the Ellman method (8) after cleaving the thioester bond with hydroxylamine at pH 7. The concentration of 3-ketodecanoyl-CoA was determined by recording the oxidation of NADH in the presence of 3-hydroxyacyl-CoA dehydrogenase. The purification of 3-ketoacyl-CoA thiolase from pig heart and the preparation of rabbit immunoglobulins against 3-ketoacyl-CoA thiolase from pig heart and against acetoacetyl-CoA thiolase from *Escherichia coli* have been described previously (9). Pig heart mitochondria were isolated as described for beef heart mitochondria (10). An acetone powder of pig heart mitochondria was prepared according to the Dahlen and Porter procedure (3).

Methods

Thiolase activity was routinely measured as described in principle by Lynen and Ochoa (11) and as detailed by Staack et al. (9). 3-Ketodecanoyl-CoA served as a substrate. 3-Hydroxyacyl-CoA dehydrogenase was assayed by measuring the oxidation of NADH in the presence of acetoacetyl-CoA or 3-ketodecanoyl-CoA as previously described (12). Enoyl-CoA hydratase was measured spectrophotometrically at 263 nm. A typical assay mixture contained in a total volume of 0.6 ml: 30 μmol of glycylglycine (pH 7.8), 1.4 μmol of 2-mercaptoethanol, 21 nmol of *d,l*-3-hydroxydecanoyl-CoA and enzyme to give a ΔA/min of 0.01 to 0.02. The acetyl-CoA-dependent fatty acid

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elongation was determined by measuring the incorporation of [1-^{14}C] acetyl-CoA into ether extractable fatty acids at 38 C. The reaction mixture contained in a total volume of 1 ml: 40 μmol of glycylglycine (pH 7.8), 130 nmol of octanoyl-CoA, 65 nmol of [1-^{14}C] acetyl-CoA (6000 dpm/nmol), 0.56 μmol of NADH and 25 μmol of rotenone. The reaction was started by the addition of an acetone powder extract containing 65 μg of protein. The extract was prepared by sonicating a suspension of acetone powder from pig heart mitochondria for 30 sec at 0 C with a Branson sonifier (equipped with a microtip) in 0.05 M glycylglycine (pH 7.8) containing 2.8 mM mercaptoethanol. Reactions were terminated after 15 min by the addition of either KOH or HCl to give a final concentration of 3 M. Termination with HCl permitted the separate extraction of free fatty acids, followed by the extraction of CoA-bound fatty acids after alkaline hydrolysis for 1 hr at 90 C. After washing the ether extracts with water, the solvent was removed in vacuum and the residues were counted in a liquid scintillation counter. In experiments where 3-ketoacyl-CoA thiolase was removed from the acetone powder extracts by immunoprecipitation, the acetone powder extracts were first centrifuged at 31,000 x g for 30 min and were then reacted with 0-400 μg of antibodies per 140 μg of extracted protein. After 1 min at room temperature, 0.05 M glycylglycine (pH 7.8) containing 10 mM 2-mercaptoethanol and bovine serum albumin (0.18 mg/ml) was added to a final volume of 1 ml. The solution was centrifuged at 31,000 x g for 30 min and the resulting supernatant was immediately assayed for thiolase and fatty acid elongation activities. Units of enzyme activity are expressed as μmol of substrates used or as products formed per min. Protein concentrations were determined by the method of Lowry et al. (13).

RESULTS AND DISCUSSION

We have studied mitochondrial fatty acid elongation with an acetone powder extract of pig heart mitochondria because this elongation system is soluble and has recently been investigated by Hinsch et al. (14), who demonstrated (a) it catalyzes most efficiently the acetyl-CoA-dependent elongation of medium chain fatty acyl-CoA's and (b) requires as an electron donor only NADH. Similar results have been obtained by Dahlen and Porter with an acetone powder extract of beef heart mitochondria (3).

TABLE I

Fatty Acid Elongation with an Acetone Powder Extract of Pig Heart Mitochondria[a]

Exp.	Additions	Deletions	Spec. activity	Rel. activity
			mU/mg of protein	%
No.1	none	none	1.00	100
	none	octanoyl-CoA	0	0
	9.7 mU thiolase	none	1.34	134
	19.5 mU thiolase	none	1.62	162
	39.2 mU thiolase	none	1.91	191
	404 mU thiolase	none	2.66	266
No.2	none	none	0.587	100
	94 μg Ab to thiolase	none	0.016	2.7
	malonyl-CoA	acetyl-CoA	0.02	3.4
	malonyl-CoA + 94 μg Ab to thiolase	acetyl-CoA	0.015	2.6
No.3[b]	none	none	0.211	100
	47 μg Ab to thiolase	none	0	0
	43 μg Ab to thiolase + 91 mU thiolase	none	0.301	143
	43 μg Ab to thiolase + 182 mU thiolase	none	0.455	216

[a]Experiments were performed as described in Experimental Procedures.

[b]Each assay contained 200 μg of protein as compared to 65 μg used in experiments No. 1 and 2 in order to obtain maximal amounts of product. However, with 200 μg of protein, the reactions were not linear for 15 min; thus the specific activities are lower than those obtained with 65 μg of protein.

Table I shows the elongation activity of the acetone powder extract was between 0.2 and 1 nmol of acetyl-CoA incorporated per min and mg of extractable protein. In the absence of octanoyl-CoA, no ether-extractable fatty acids were formed; the system thus is not capable of de novo fatty acid synthesis. In agreement with previous reports, malonyl-CoA could not substitute for acetyl-CoA in the elongation reaction (15). The slight incorporation of malonyl-CoA into fatty acids most likely resulted from the presence of small amounts of acetyl-CoA in the malonyl-CoA preparation.

The removal of 3-ketoacyl-CoA thiolase—which acts on substrates of various chain lengths—from the acetone powder extract by immunoprecipitation resulted in a nearly complete inhibition of the fatty acid elongation system (see Table I). When 3-ketoacyl-CoA thiolase was first removed from the extract by immunoprecipitation and then replaced by purified 3-ketoacyl-CoA thiolase, the elongation activity was reconstituted (see Table I). The results of a detailed inhibition study of both 3-ketoacyl-CoA thiolase and the fatty acid elongation system by antibodies to 3-ketoacyl-CoA thiolase are presented in Figure 1. This data shows that antibodies to 3-ketoacyl-CoA thiolase, in contrast to antibodies against acetoacetyl-CoA thiolase from *E. coli*, inhibited thiolase and the fatty acid elongation system in parallel, a finding that suggests 3-ketoacyl-CoA is a component enzyme of the mitochondrial fatty acid elongation system and possibly catalyzes the rate-limiting step in this pathway. The small thiolase activity not precipitated at optimal antibody concentrations was most likely the cause for the residual fatty acid elongation activity. Since this experiment suggests the thiolase-catalyzed condensation of octanoyl-CoA and acetyl-CoA is the rate-limiting step of the elongation reaction, the effect of added purified 3-ketoacyl-CoA thiolase on the rate of fatty acid elongation was investigated. Table I shows the elongation activity was stimulated by the addition of 3-ketoacyl-CoA thiolase, a finding which supports the conclusion concerning the rate-limiting step of the pathway. To further substantiate this conclusion, the individual reactions of the fatty acid elongation system (except for the NADH-dependent enoyl-CoA reductase) were measured. The latter enzyme could not be measured because 3-hydroxyacyl-CoA dehydrogenase is present in the acetone powder extract at much higher levels and interferes with both the spectrophotometric and radioactive assay of the NADH-dependent enoyl-CoA reductase. The activities of thiolase,

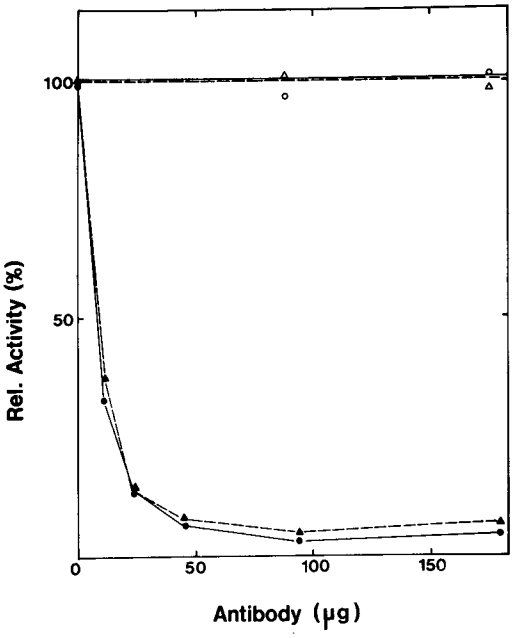

FIG. 1. Effects of antibodies to 3-ketoacyl-CoA thiolase on the activities of 3-ketoacyl-CoA thiolase and of the mitochondrial fatty acid elongation system. Experiments were performed as described in Experimental Procedures. Thiolase activity (▲) and fatty acid elongation activity (●) after addition of antibodies prepared against 3-ketoacyl-CoA thiolase. Thiolase activity (△) and fatty acid elongation activity (○) after addition of antibodies prepared against *E. coli* acetoacetyl-CoA thiolase.

3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase in the direction of fatty acid elongation are listed in Table II. These approximate values indicate again that the rate of fatty acid elongation is determined by the rate of the thiolase-catalyzed condensation reaction. The lower values of the condensation reactions, as compared to the elongation activities, probably result from differences in conditions at which the two types of assays were performed.

Analyses of the elongation products revealed that ca. 50% of the fatty acids were present as free acids; the free and CoA-bound fatty acids were found to be mostly hydroxy acids. The ratios of hydroxy acids to saturated and unsaturated acids were ca. 3:1 and thus were similar to those observed by Hinsch et al. (14). The high percentage of hydroxy acids, which is typically observed with mitochondrial extracts of heart, skeletal muscle and other extrahepatic tissues, but not with liver mitochondria (14,16), points to enoyl-CoA reductase as the rate-limiting enzyme in the formation of saturated fatty acids. We conclude that the condensation reaction catalyzed by 3-ketoacyl-

TABLE II

Activities of the Fatty Acid Elongation System and of Several of Its Component Enzymes in a Homogenate and Acetone Powder Extract of Pig Heart Mitochondria

Enzyme[a]	Specific activity	
	Homogenate	Acetone powder
	mU/mg of protein	
Fatty acid elongase	0.88	0.37
3-Ketoacyl-CoA thiolase[b]	0.5	0.25
3-Hydroxyacyl-CoA dehydrogenase[c]	586	173
Enoyl-CoA hydratase	34	18

[a]Enzyme assays are described in Experimental Procedures.

[b]Thiolase activities were measured in the cleavage direction with 3-ketodecanoyl-CoA as substrate. The ratio of activities in the cleavage vs the condensation direction of 210 determined with purified thiolase was used to calculate the listed values.

[c]3-Hydroxyacyl-CoA dehydrogenase was assayed with acetoacetyl-CoA as a substrate. An activity ratio of 2 determined for the reduction of acetoacetyl-CoA vs that of 3-ketodecanoyl-CoA with pure enzyme was used to calculate the listed values.

CoA thiolase is the rate-limiting step in heart mitochondria when the incorporation of acetyl-CoA into all ether-extractable fatty acids (including 3-hydroxy acids and enoic acids) is measured, whereas the reduction of the enoyl-CoA intermediates is possibly the rate-limiting step in the formation of saturated fatty acids. The parallel and virtually complete inhibition of 3-ketoacyl-CoA thiolase and of the fatty acid elongation activity by antibodies to 3-ketoacyl-CoA thiolase provides proof that fatty acid elongation in heart mitochondria occurs by reversal of fatty acid oxidation. Since enoyl-CoA reductase is membrane-bound (2) and since a significant fraction of the β-oxidation enzymes remains membrane-associated even when mitochondria are broken (H. Staack and H. Schulz, unpublished observation), the fatty acid elongation is best characterized as membrane-associated. Because of the well known location of the enzymes of fatty acid oxidation within the space surrounded by the inner mitochondrial membrane (17), the fatty acid elongation system is assumed to be associated with the inner mitochondrial membrane. In view of our conclusion about the enzymes of the mitochondrial fatty acid elongation system in heart, we suggest the observed behavior of the elongation activity as a single protein with a molecular weight of 135,000 (3) may reflect the molecular weight range in which the four component enzymes of the system are present in optimal amounts.

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Incorporation of [1-^{14}C]Octadecanol into the Lipids of *Leishmania donovani*

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ABSTRACT

After incubation of stationary phase *Leishmania donovani* with [1-^{14}C] octadecanol, about 70% of the precursor was taken up within 3 hr. Wax esters and acyl moieties of glycerolipids contained most of the ^{14}C-activity from 3 to 6 hr, because octadecanol was partly oxidized to stearate. Ether moieties were only weakly labeled. After 40 hr, 1-0-alkyl and 1-0-alk-1′-enyl diacylglycerols as well as 1-0-alkyl and 1-0-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamines contained nearly all of the radioactivity. Most of the label in the neutral ether lipids was located in the alkyl ether side chain, whereas, in the phosphatidylethanolamine fraction, most of the label was found in the alkenyl ether side chain. Administration of 1-0-[1-^{14}C] hexadecyl glycerol resulted in rapid labeling of the vinyl ether side chain of phosphatidylethanolamine plasmalogen (1 hr) increasing further at 2.5 hr. Most of the radioactivity in the alkoxy diacylglycerols was found in the 1-0-alkyl moiety.

INTRODUCTION

As stated by Thompson and Nozawa (1), ether lipids are characteristically found in protozoa. Hack et al. (2) examined five species of trypanosomatid flagellates including *Leishnania donovani* and found all of them gave a positive reaction for ethanolamine plasmalogen staining on thin layer plates. Considerable work on lipid metabolism has been done with the ciliate, *Tetrahymena pyriformis*, which contains 1-0-alkyl glycerophospholipids but no plasmalogens (3). Ether analogues of triacylglycerols have not been reported.

In the promastigote form of the haemoflagellate, *L. donovani*, cultured in a lipid-free medium, the main phospholipids are phosphatidylcholine (52%) and phosphatidylethanolamine (23%). Whereas the former contains only traces of plasmalogens, 56% of the latter exists in the plasmalogen form and 2.6% in the 1-0-alkyl-2-acyl form. In addition, 1-0-alkyl and 1-0-alk-1′-enyl diacylglycerols (1.2% and 0.7% of neutral lipids, respectively) are found in *L. donovani* (4). This demonstrates these protozoans are able to synthesize ether lipids from basic metabolites like glucose or amino acids and that they are not dependent on the uptake of direct precursors, such as long chain alcohols, from the host. The occurrence of neutral and polar plasmalogens in this parasite suggests an investigation of their biosynthesis in this organism would be of interest.

This paper describes the synthesis of alkoxy lipids after administration of a long chain alcohol, the incorporation into individual lipids and the distribution of radioactivity in the aliphatic side chains. In addition, the metabolism of radioactive 1-0-hexadecyl glycerol is investigated.

MATERIALS AND METHODS

Cell Culture and Incubations

L. donovani, LRC-51, was obtained from the Bernhard-Nocht-Institut für Schiffs- und Tropenkrankheiten, Hamburg. Organisms have been maintained at 27 C in a modified lipid- and protein-free Nakamura medium (5) since 1971 with serial transfers every 7 days. For incubation, cells were obtained from 16 Erlenmeyer flasks (2 ℓ), containing 1 ℓ medium after inoculation. Cells were harvested at the end of the logarithmic phase of growth by centrifugation (2000 g), pooled and checked for viability by phase contrast microscopy. Cell counting was performed by means of a haemocytometer (improved Neubauer type). For turnover experiments, 1.0 to 4.4 x 10^{10} cells were grown for various times at room temperature (22 C) with a cell density of 4.5 x 10^9 cells/ml in 0.9% NaCl/Nakamura medium (1:1, v/v) yielding 12 to 51 μmol lipid phosphorus. For incubations longer than 16 hr, a density of 3 x 10^9 cells/ml was used. Incubation vessels were round bottom flasks equipped with magnetic stirrers. Cell numbers did not change under these conditions. [1-^{14}C] Octadecanol (7.4 mCi/mmol, Amersham Buchler, Buckinghamshire, U.K.) was dissolved in absolute ethanol and added dropwise with a Hamilton syringe. The concentration of octadecanol was kept between 0.15 and 0.30 mmol/ℓ. The pH was near neutral and changed slowly toward growth optimum (pH 7.3) during incubation. For experiments lasting 142 hr, the inoculum was 2 x 10^5 cells/ml in two Erlenmeyer flasks (2 ℓ), with 680 ml fresh Nakamura medium each. The concentration of [1-^{14}C] octadecanol was 0.4 μmol/ℓ. 1-0-[1-^{14}C] Hexadecyl glycerol (56

mCi/mmol) was prepared according to Baumann and Mangold (6) and used for incorporation studies (1 x 10^8 cell/ml PBS-buffer, 0.008 μmol 0-alkyl glycerol/ml). Incubations were stopped by extracting the lipids using the procedure of Folch et al. (7). Lipids were stored in chloroform under nitrogen at -20 C.

Measurement of Radioactivity

Radioactivity was measured in a Packard Tricarb Model 3320 liquid scintillation spectrophotometer. Quench was controlled by use of an external standard. Counting efficiency was approximately 82% for ^{14}C. The uptake of [1-^{14}C]octadecanol was measured by taking two separate 100 μl samples from an incubated cell suspension (3 x 10^9 cells/ml). The samples were diluted with 5 ml PBS-buffer, centrifuged (1000 g), washed a second time and extracted using the Dawson and Kemp procedure (8). The chloroform-methanol mixture was dried in a counting vial and dissolved in 15 ml Riasolve (Koch-Light Lab., Colnbrook, Bucks., U.K.). Recoveries of ^{14}C-activity were in excess of 95% for all incubation periods. Radioactivity in the upper Folch phase was always less than 2% of the activity incorporated into lipids indicating little degradation into water-methanol soluble compounds such as peptides, amino acids or TCA cycle acids. Proteins were not labeled at all. ^{14}C-activity of carbon dioxide was not determined.

Analysis of Lipids

Aliquots of lipid extracts were taken for determination of total recovered ^{14}C activity and the individual lipids were analyzed for distribution of radioactivity by subjecting aliquots of the total lipids (1 to 5 x 10^5 dpm) to two-dimensional thin layer chromatography (TLC) on Silica Gel HR 60 (Merck, Darmstadt, Germany). The first solvent system consisted of hexane/diethyl ether (70:30, v/v) and the second of hexane/diethyl ether (95:5, v/v). Additional one-dimensional runs were done using hexane/diethyl ether/acetic acid (50:50:1, v/v) on Silica Gel G. Phospholipids were run on high performance thin layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) with the solvent systems chloroform/methanol/25% NH_3 (65:35:8, v/v) and chloroform/methanol/acetic acid (50:25:8, v/v) (9). Lipids were identified as previously described with standards prepared from rabbit or ox erythrocytes (4). Solvents were reagent grade (Merck, Darmstadt, Germany). Diethyl ether was distilled before use. Lipids were visualized by exposure to iodine vapor, spots marked with a needle and the plates left overnight to eliminate iodine. Spots were scraped into scintillation vials, the silica gel was deactivated by addition of 0.5 ml water and 15 ml Riasolve was added.

For analysis of aliphatic chains, aliquots of the total lipids representing 1-5 μmol lipid phosphorus were used. Neutral lipids were separated from phospholipids by preparative TLC on Silica Gel HR 60 using hexane/diethyl ether (40:60, v/v) as developing solvent. Acetic acid was omitted whenever plasmalogens were present and nitrogen was used for all operations. Triacylglycerols, ether analogs of triacylglycerols and sterol esters were eluted with chloroform/methanol (2:1, v/v) and diethyl ether/methanol (10:1, v/v) and separated on Silica Gel HR 60 with hexane/diethyl ether (90:10 or 95:5, v/v) (9). Phospholipids were separated into the major phospholipid classes by TLC on Silica Gel HR 60 with chloroform/methanol/water (70:30:5, v/v) (10). Visualization of single fractions was accomplished by exposure of standards to iodine vapor. Phospholipids were eluted as reported elsewhere (10,11). Single phospholipid classes were purified by TLC with chloroform/methanol/25% NH_3 (65:35:8, v/v).

1-0-Alkyl- and 1-0-alk-1′-enylglycerols were prepared from neutral and polar ether lipids by reduction with vitride [$Na_2AlH_2(OCH_2CH_2OCH_3)_2$ 70% in benzene] following the procedure of Snyder et al. (11), as modified by Blank et al. (12). The separation of reaction products was performed on HPTLC plates with diethyl ether/water (100:0.5, v/v). Recovery percentage from ^{14}C-labeled ethanolamine phosphatides as reported by Blank et al. (90%, 92%) (12) were not achieved in the course of our work (70-85% for phosphatidylethanolamine, 94-99% for the neutral ether lipid). Spraying with the 2,4-dinitrophenylhydrazine reagent indicated occurrence of condensation products running just ahead of the fatty alcohols (13,14).

As the method of choice, aldehydogenic moieties were converted into the corresponding dimethyl acetals (DMAs) from plasmalogens with BF_3 methanol (15). Separation of simultaneously formed 1-0-alkylglycerols and fatty acid methyl esters (FAMEs) was carried out on HPTLC plates (10 x 20 cm) by double development, first with dichloroethane (15 cm) (16) and second with diethyl ether/water (100:0.5, v/v) (8 cm) in order to move the 1-0-alkylglycerols away from the origin. FAMEs were checked for absence of aldehydes (17). Recovery of ^{14}C-activity for phosphatidylethanolamine was between 85% and 95%, and for the neutral ether lipid more than 95%.

The sterol ester/wax ester fraction was treated according to the Morrison and Smith method (15). Fatty alcohols, sterols and FAMEs were separated on silica gel using hexane/diethyl ether/acetic acid (50:50:1, v/v). Aliquots of the total lipids were taken for determination of lipid phosphorus which was performed by the Broekhuyse method (18). Results are presented as dpm per cell number (promastigotes).

Gas-Liquid Chromatography

FAMEs were analyzed on a 2 m x 3 mm coiled glass column packed with 3% EGSS-X on Gas Chrom Q, 100-120 mesh, long chain alcohols on Apolar 10 C (Applied Science Lab., Inc., State College, PA), and DMAs on 3% EGSS-X and Apolar 10 C. The instrument was a Packard, model 472. The column temperature was 165 C or 185 C (FAMEs), 140 C or 170 C (DMAs) and 190 C (fatty alcohols). Peaks were identified by comparison of retentions times with known standards (NuChek Prep, Inc., Elysian, MN). In addition, DMAs were analyzed by GC/MS analysis (Varian MAT CH 7, Bremen, Germany). Authentic dimethyl acetals of hexadecanal and octadecanal gave the same analytical results as the two main components of DMAs from phosphatidylethanolamine. Quantification was done by multiplication of peak height with retention time using various internal standards (15).

RESULTS

Uptake and Incorporation of ^{14}C-Octadecanol

The uptake of [1-^{14}C]octadecanol into the cells of *L. donovani* was complete after 3 hr (Fig. 1). No more than 70% of the precursor was taken up if it was administered in absolute ethanol. In comparison, [1-^{14}C]octadecanol entrapped within liposomes (prepared according to Black et al. [19]) entered cells nearly to completeness by 5 hr. The incorporation of the fatty alcohol into lipids was slow for most lipid classes in contrast to the rapid uptake. After 3 hr only 3% of the absorbed labeled fatty alcohol was used for lipid synthesis while 97% must have been deposited in the cells; 53% of the incorporated radioactivity was present in wax esters - the major component of neutral lipids (32.5% of neutral lipids) besides sterols and triacylglycerols.

Incubations of 6 and 16 hr showed phosphatidylcholine and phosphatidylethanolamine to contain most of the radioactivity (Fig. 2). At 24 hr, wax esters were labeled by 49% and there was also a further increase of label in the other lipids except for phosphatidylcholine

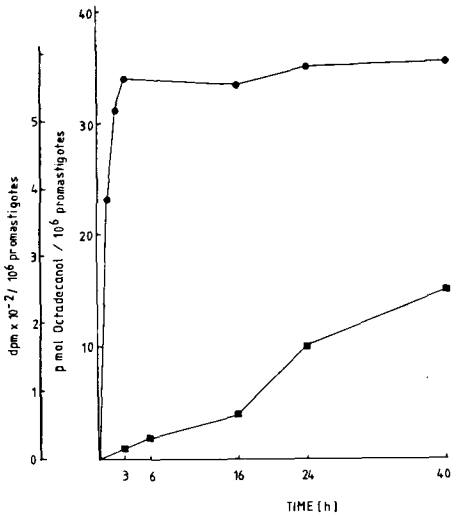

FIG. 1. Uptake anad incorporation of [1-^{14}C]-octadecanol by *L. donovani* promastigotes. Symbols: ●, uptake into cells; ■, incorporation into lipids other than free fatty alcohols. One biological sample was incubated for each time period as described in Materials and Methods. Each value in Figs. 1-4 represents an average of at least two separate determinations which usually agreed within ± 5% for major components and ± 15% for minor ones. The unit dpm x 10^{-2}/10^6 promastigotes is the same in Figs. 1 and 2.

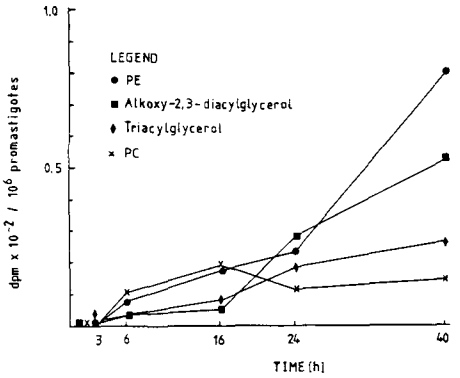

FIG. 2. Time course of incorporation of [1-^{14}C]-octadecanol into various lipids of *L. donovani:* ■, alkoxy-2,3-diacylglycerols; ●, phosphatidylethanolamine fraction; X, phosphatidylcholine; ♦, triacylglycerols.

relative to the 16 hr incorporation. Still later, the ^{14}C-incorporation into the phosphatidylethanolamine fraction and the neutral ether lipids was increased while the wax ester activity diminished. Free alkyl and alk-1-enyl glycerols were labeled to a minute extent for all periods.

Oxidation of ^{14}C-Octadecanol

Methanolysis of individual lipids revealed the

labeling pattern was determined not only by incorporation of octadecanol but also by oxidation of octadecanol to stearic acid. The percentages of the radioactivity in total lipids of *L. donovani* present in acyl residues were 1.2%, 1.4%, 5.1% and 7.5% at 3, 6, 16 and 40 hr, respectively. No more than 0.2% of the total recovered radioactivity was associated with free fatty acids. At the same time, the fatty acid composition of phosphatidylethanolamine was significantly changed by the administration of octadecanol (Fig. 3). The percentage of stearate as well as of palmitate increased up to 6 hr, indicating hexadecanol, the main fatty alcohol of *L. donovani* promastigotes (84% of fatty alcohols), took part in the oxidation following the uptake of 35 pmol octadecanol/10^6 promastigotes, which was equivalent to a 15% increase of the intracellular fatty alcohol concentration. Palmitate apparently was desaturated to palmitoleate, whereas stearate was desaturated to yield oleate and linoleate, both accumulating at 6 and 16 hr, respectively. Thus, at 16 hr, the fatty acid composition of PE equaled that of untreated cells (0 hr).

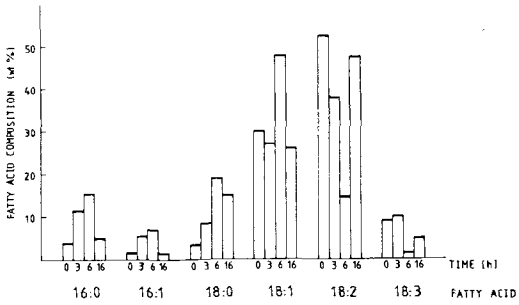

FIG. 3. Time course of fatty acid composition of the PE fraction after incubation with [1-^{14}C]octadecanol (0.4 mmol/ℓ) for 3, 6 and 16 hr (only 16:0 to 18:3 fatty acids are shown representing more than 90% of total fatty acids). Each value is the mean of two separate determinations except for those of 0 hr, which were done from five different cultures.

Distribution of Radioactivity in Aliphatic Side Chains

The distribution of ^{14}C-activity at various periods in the methanolysis products of the sterol/wax ester fraction showed that the acyl and alcohol moieties were labeled to a similar extent from 3 to 16 hr. The extensive labeling of wax esters at 24 hr was caused mainly by the alcohol moiety (77%). Compared to the fatty acid labeling, radioactivity in sterols was negligible (<2%). From this, we concluded that β-oxidation and recycling of C-2 units were low.

The distribution of radioactivity in the aliphatic moieties of the phosphatidylethanolamine fraction and alkoxy-2,3-diacylglycerols is shown in Figure 4. For the latter, a sizeable proportion of ^{14}C-activity was incorporated into the acyl moiety during the first 3 to 6 hr while the alkyl and alkenyl residues gained radioactivity more slowly. By 40 hr, the alkyl group contained 61%; the alk-1-enyl group contained 24% of the alkoxy-2,3-diacylglycerol radioactivity. In the phosphatidylethanolamine fraction, the labeling of the alkyl moiety preceded that of the alk-1-enyl moiety at 3 and 6 hr, representing 65% and 42% of the ^{14}C activity of this lipid whereas incubations of 16 and 40 hr showed the alk-1-enyl group was labeled to a degree of 51% and 84%, respectively. In phosphatidylcholine, more than 84% of ^{14}C activity was located in the acyl group for every time interval. The identity of the alk-1-enyl side chains of phosphatidylethanolamine and alkoxy diacylglycerols was confirmed by methanolysis. The resulting dimethyl acetals with saturated C_{16}-C_{18} chains were isolated by TLC and gas liquid chromatography/mass spectrometry (GLC/MS).

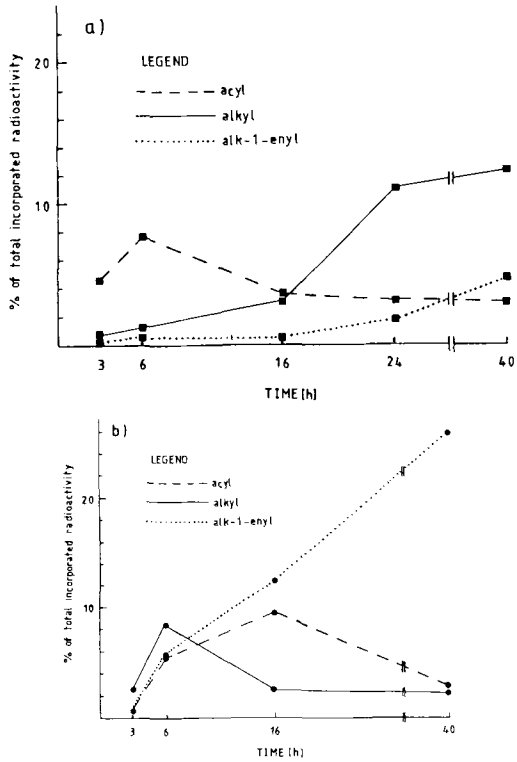

FIG. 4. Distribution of ^{14}C-activity in acyl, alkyl and alk-1-enyl moieties of (a) alkoxy-2,3-diacylglycerols; (b) the phosphatidylethanolamine fraction at various incubation periods. Values are percentages of total lipid radioactivity other than fatty alcohols.

Incubation with ^{14}C-Octadecanol for One Growth Cycle

Incubations lasting from inoculation until late logarithmic phase of growth (142 hr, Nakamura medium) were carried out with one-thousandth of the fatty alcohol concentration used in the incubations described above (Table I). More than 66% of the recovered label was found in the phosphatidylethanolamine fraction and no more than 1.7% in the free fatty alcohols. Transesterification showed 47% of the total incorporated activity was located in the alk-1-enyl moiety of phosphatidylethanolamine. The alkoxy diacylglycerols represented only 2.6% of the total activity, half of it manifesting the plasmalogen form. In the phosphatidylcholine fraction, ^{14}C-activity of the alk-1-enyl group was nearly twice as high as that in the neutral plasmalogen, but most of the radioactivity (85%) was found in the acyl residues.

Uptake of ^{14}C-Hexadecylglycerol

In order to clarify the way 1-0-alk-1'-enyl glycerolipids are synthesized, 1-0-[1-^{14}C] hexadecyl glycerol was incubated with *L. donovani* for three different time intervals (1, 2.5 and 5 hr). Each experiment was duplicated. The precursor was taken up as fast as octadecanol ranging up to 73% at 2.5 hr. Table II shows the distribution of radioactivity in the aliphatic moieties of phosphatidylethanolamine and alkoxy diacylglycerols. Again, acyl groups were labeled to some extent, but in contrast to incubations with octadecanol, the alkenyl group of the phosphatidylethanolamine fraction gained most of the activity from the beginning. In the alkoxy diacylglycerols, this was the case for the alkyl group up to 2.5 hr. At 5 hr, the label in the alkyl group had decreased considerably, indicating a rapid turnover. The phosphatidylcholine fraction, which was labeled twice as much as phosphatidylethanolamine by 5 hr, contained most of the radioactivity in the acyl residues (> 95%).

DISCUSSION

Food ingestion by *L. donovani* probably takes place in the reservoir area through pinocytic vesicles that fuse together with primary lysosomes, forming multivesicular bodies where the final digestion occurs (20). The way the precursor is made available for the cell during this process might influence the mode of its incorporation into lipids, since the uptake of the precursor was complete after 3 hr, but incorporation of ^{14}C-activity into lipids was very low at that time. Since *Leishmania* is known to contain rather large lipid inclusions (Koyama and Ilardi [21] and Rudzinska et al. [22]) the absorbed fatty alcohol was possibly deposited in those droplets and used extensively from 24 hr on.

Labeling of lipids over time by [1-^{14}C] octadecanol was influenced by part of the fatty alcohol's oxidation to stearate, which was incorporated quickly. The long chain alcohol

TABLE I

Incorporation of [1-^{14}C] Octadecanol into Lipids and Aliphatic Moieties of the Phosphatidylethanolamine Fraction, Alkoxy-2,3-Diacylglycerols and Phosphatidylcholine after Incubation of *L. donovani* in Nakamura Medium for 142 hr [a]

	dpm/10^6 promastigotes
Neutral lipids	271.5
Wax esters/sterol esters	19.5
Alkoxy diacylglycerols	63.5
Triacylglycerols	73.3
Fatty alcohols	41.5
Total phospholipids	2225.6
Phosphatidylethanolamine	1492.7
Phosphatidylcholine	466.6

	Alkoxy diacylglycerols	Phosphatidylethanolamine	Phosphatidylcholine
Alkenyl	33.4	1143.0	55.7
Alkyl	16.3	55.2	15.7
Acyl	12.6	149.6	389.1

[a] All figures represent the mean values from duplicate incubations. The duplicate values varied by less than 8%.

itself was incorporated into ether lipids only at moderate rates. It is suggested that the enzymatic equilibrium ROH⇌FA takes part in the regulation of ether lipid synthesis as pointed out by Malins and Sargent (23) and Kapoulas and Gabrielides (24). Clear cut differences resulted when octadecanol was administered in concentrations in mM or μM, respectively, since in the former case fatty acid, wax ester and alkoxy diacylglycerol production was high, whereas in the latter most of the label was found in the ether moieties of the phosphatidylethanolamine fraction indicating ether-linked aliphatic side chains of glycerolipids are synthesized from long chain alcohols. This has been shown for various tissues (23,25-33), as well as for *Tetrahymena pyriformis* (34). Only saturated alkyl moieties with 16-18 and 20 carbon atoms occur in ether lipids, wax esters and free alcohols of *L. donovani*, even though lipids of these protozoa contain mainly unsaturated acyl side chains (79.5% of total fatty acids). This indicates production of saturated alcohols is highly favored over that of unsaturated alcohols. Administered unsaturated alcohols (18:1, 18:2, 18:3), medium chain alcohols (12:0, 14:0), eicosanol and docosanol were esterified to form wax esters, but were not incorporated into ether side chains (unpublished observations) pointing toward a high specifity of ether bond formation. These results agree with Rock et al. (35) who recently suggested the composition of alkyl ether moieties is controlled at the level of fatty alcohol synthesis in rabbit haderian gland. For rat brain ether lipids, Natarajan and Schmid (36) pointed out substrate specifity in formation of both fatty alcohol and alkyl dihydroxy-acetone phosphate controls the composition of alkoxy moieties.

Following the administration of [1-^{14}C]-octadecanol, the ratio of radioactivity in alkenyl side chains to alkyl side chains of phosphatidylethanolamine was 0.3, 0.7, 5.0, 11.9 and 20.7 by 3, 6, 16, 40 and 142 hr, respectively, whereas the molar ratio of these moieties in untreated cells is 21.4. We conclude from this that a precursor-product relationship of the alkyl and alkenyl side chains exists in phosphatidylethanolamine as shown for various organisms in vivo (37-39) and for different cell-free systems in vitro (11,40,41). In order to test this assumption, 1-0[1-^{14}C]hexadecyl glycerol was incubated with *L. donovani*. The vinyl ether residue of phosphatidylethanolamine was labeled highly after 1 hr (6.3% of total incorporated radioactivity) and increased strongly up to 2.5 hr (20.4%). The proportion of radioactivity in the alkyl ether moiety of phosphatidylethanolamine remained small indicating rapid formation of the alk-1-enyl moiety. Cleavage of the glyceryl ether occurred to some extent as observed with other protozoa

TABLE II

Uptake of 1-0-[1-^{14}C]Hexadecyl Glycerol into *L. donovani* Promastigotes, Incorporation into Lipids other than Alkylglycerols and Distribution of ^{14}C-Activity in Alk-1-enyl, Alkyl and Acyl Moieties of the Phosphatidylethanolamine Fraction and Alkoxy-2,3-diacylglycerols after Incubation of 1, 2.5 and 5 hr [a]

	dpm/10^6 promastigotes		
Time (hr)	1	2.5	5
Uptake	6439.4	6634.1	6971.3
Incorporation	1609.8	2938.9	3903.9
Phospholipids/Alkoxy diacylglycerols	1.5	2.6	3.8
Phosphatidylethanolamine			
Alkenyl	101.6	598.5	793.9
Alkyl	11.7	25.7	34.7
Acyl	18.3	317.0	144.0
Alkoxy diacylglycerols			
Alkenyl	13.3	94.9	119.4
Alkyl	432.9	1376.6	1049.5
Acyl	87.0	117.2	74.8

[a]Values given are means of duplicate determinations of duplicate biological samples from which aliquotes were removed after the indicates times. Individual values deviates less than 10% from the mean.

(39,42) and labeled acyl groups were incorporated into lipids, but they apparently did not take part in the formation of alk-1-enyl moieties as they were incorporated mainly into phosphatidylcholine.

The alkyl chain of alkoxy diacylglycerol was labeled greatly by 1-0-[1-^{14}C]hexadecyl glycerol with a maximum at 2.5 hr. Therefore, direct acylation of the alkyl glycerol must occur. The ratio of ^{14}C-activity in phospholipids to alkoxy diacylglycerols changed from 1.5 after 1 hr to 3.8 by 5 hr, pointing to a close connection of phospholipid synthesis and alkoxy diacylglycerol metabolism, since lipase action on 1-0-alkyl-2,3-diacylglycerol can produce 1-0-alkyl-2-acyl-glycerol - a possible precursor of glycerophospholipids. For *T. pyriformis*, it has been demonstrated that triacylglycerols can be precursors of phospholipids (43) and that the phospholipid synthesizing system in microsomes is closely coupled to the triacylglycerol synthesizing system (44). In contrast to the fast turnover of label in the alkyl chain of alkoxy-2,3-diacylglycerols, radioactivity in the alk-1-enyl moiety accumulated slowly, as observed in the octadecanol incubation. From our data, we conclude, as proposed by Snyder (45), that 1-0-alkyl-2,3-diacylglycerol is not directly converted to 1-0-alk-1′-enyl-2,3-diacylglycerol. The latter probably originates from cleavage products of phosphatidylethanolamine plasmalogen like 1-0-alk-1′-enyl-2-acylglycerol, which was shown to be acylated to neutral plasmalogen in tumor tissue by Blank et al. (46).

ACKNOWLEDGMENT

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Activation of the Phospholipase A_1 Activity of Lipoprotein Lipase by Apoprotein C-II

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ABSTRACT

The effect of apo very low density lipoprotein (apo VLDL) and apoprotein C-II on the phospholipase A_1 activity associated with lipoprotein lipase (E.C.3.1.1.3) was studied using purified bovine milk lipoprotein lipase. The enzyme degraded ^{14}C phosphatidylcholine (PC) to ^{14}C 2-acyl lysophosphatidylcholine at a rate of 0.28 ± 0.01 nmol/min/ml and triolein at a rate of 20.3 ± 0.4 nmol/min/ml in mixed emulsions of PC and triolein. The phospholipase activity and triacylglycerol lipase activity were both increased by the addition of apo VLDL and apoprotein C-II. After maximal activation, the rate of PC degradation was 1.19 ± 0.02 nmol/min/ml and triolein degradation 64.4 ± 0,4 nmol/min/ml. Activation of phospholipase A_1 activity and triacylglycerol lipase activity occurred in parallel.

INTRODUCTION

The main function of lipoprotein lipase (E.C.3.1.1.3) is the catabolism and clearance from the circulation of chylomicrons and very low density lipoproteins by hydrolysis of their triacylglycerols (1).

Formation of triacylglycerol-depleted remnant particles in vivo appears to be associated with a loss of phospholipid (principally phosphatidylcholine) (2) and much of that lost is hydrolyzed to lysophosphatidylcholine. In addition, hydrolysis of phospholipid occurs when triglyceride-rich lipoproteins are incubated with post-heparin plasma (3,4). This degradation of phospholipids may result from the action of lipoprotein lipase, since it has been demonstrated that lipoprotein lipase has associated phospholipase A_1 activity. Thus, purified bovine milk lipoprotein lipase has been shown to degrade phosphatidylcholine (PC) of both chylomicrons (5) and very low density lipoprotein (VLDL) (6), and this action may account for the removal of surface phospholipid during chylomicron and VLDL catabolism.

The phospholipase A_1 activity of lipoprotein lipase may derive from the broad substrate specifity of the enzyme since it can hydrolyze the 1 or 3 acyl ester bonds of partial glycerides and soluble esters (7,8) in addition to triglycerides.

A characteristic feature of the enzyme is that it requires a cofactor, apoprotein C-II, for maximal activity toward triglycerides containing long chain fatty acids. A number of investigations have shown that, whatever the source of the enzyme, there is a several-fold increase in activity toward triglycerides in the presence of apoprotein C-II (9-14). Activation is not observed when partial glycerides and soluble esters are substrates (15). Apoprotein C-II is a constituent of triglyceride-rich lipoproteins and high density lipoprotein (16).

Since PC may be a natural substrate for the enzyme (it is the main phospholipid component of the triglyceride-rich lipoproteins), it is important to establish whether activation by apoprotein C-II also is required for maximal hydrolysis of this phospholipid. We have shown phospholipase A_1 activity associated with lipoprotein lipase of rat adipose tissue is activated by serum and VLDL (17). In addition, Groot et al. (18) have shown C-II activation of phosphatidylethanolamine (PE) degradation by rat heart lipoprotein lipase, but reported the enzyme had little activity toward PC.

In order to assess whether PC hydrolysis by lipoprotein lipase is activated by apoprotein C-II, we have determined the effect of human apoprotein C-II on PC hydrolysis by highly purified bovine milk lipoprotein lipase.

MATERIALS

Glycerol trioleate, fatty-acid-free bovine serum albumin, silicic acid, Co-Enzyme A, (CoA) adenosine 5-triphosphate and 1-acyl-2-lyso-phosphatidylcholine (ex egg yolk) were obtained from Sigma, London; 2,5-diphenyloxazole (PPO) and 1,4[di-2-(5-diphenyloxazole)] benzene (POPOP) from Koch Light Ltd.; silica gel impregnated fiber glass chromatography sheets from Gelman Hawksley; [1-^{14}C] linoleic acid (sp act 60 mCi/mmol) and glycerol tri-[1-^{14}C] oleate (sp act 55 mCi/mmol) from the Radiochemical Center, Amersham; heparin from Evans Medical Ltd., Liverpool; Sepharose 4B and Sephadex G 100 from Pharmacia, London; and DEAE-cellulose from Whatman Biochemicals. All other chemicals were obtained from BDH Ltd.

METHODS

Preparation of ^{14}C Phosphatidylcholine

1-acyl-2-[1-^{14}C] linoleyl-*sn*-glycerophosphatidylcholine was prepared by re-acylation of 1-acyl-2-lysophosphatidylcholine with [1-^{14}C]-linoleic acid using a rat liver homogenate (19). [1-^{14}C] Linoleic acid (250 μCi) was mixed with 148 mmol of lysophosphatidylcholine, 1.07 mmol ATP, and 26 mmol of CoA in 25 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) and sonicated for 2 min. The mixture was added to 25 ml of a 25% (w/v) rat liver homogenate in the same buffer and incubated for one hour at 37 C. After incubation, the homogenate was extracted with 250 ml methanol-chloroform (2:1, v/v) containing 0.1 g/l butylated hydroxyanisole and then filtered. The filtrate was mixed with 150 ml of water and 150 ml of chloroform. The chloroform phase was removed and dried under reduced pressure at 40 C. The extract was dissolved in diethyl ether and phospholipids precipitated by the addition of cold acetone. The phospholipids were redissolved in chloroform, flushed with nitrogen and stored at -20 C. The specific activities (sp act) of the preparations varied between 6.7 and 11.5 mCi/mmol. The radiochemical purity was checked by thin layer chromatography (TLC) on Silica Gel G in chloroform-methanol-ammonia (70:30:4, v/v/v). Between 98-99% of the radioactivity applied was recovered in the zone corresponding to PC.

Phosphatidycholine

PC was isolated from rat liver using the same technique as for the isolation of the labeled PC from rat liver homogenates.

Lipoprotein Lipase

Lipoprotein lipase was prepared from cow's milk by batch absorption and affinity chromatography on heparin-Sepharose (20). The heparin-Sepharose was prepared by the method of Lindahl et al. (21). The final preparation had a sp act of 28,000 mU mg protein (1 mu = 1 nmol fatty acid/min). It was diluted with 0.5 M NaCl to 200 mU/ml and then stored at -20 C.

VLDL C Apoproteins

The d $<$ 1.006 g/ml fraction was prepared from the serum of patients with type IV and type V hyperlipoproteinemia by ultracentrifugation at 40,000 rpm for 18 hr at 10 C in an MSE 8 x 25 ml angle head rotor (22). The VLDL was delipidated with ethanol-ether (3:1, v/v) (23). Tris soluble apoproteins were prepared by suspension of apo VLDL in 0.2 mmol/l tris-HCl, pH 8.1 (24). Individual C apo proteins were prepared by solubilization of apo VLDL in 0.2 mmol/l sodium dodecyl sulphate followed by gel filtration on Sephadex G-100. After gel filtration, the C apo proteins were separated by DEAE-cellulose chromatography in 8 mmol/l urea (24). The purity of the apoproteins was checked by polyacrylamide gel electrophoresis in 8 mol/l urea (25). All preparations gave a single band.

Substrates for Phospholipase A$_1$ and Lipoprotein Lipase

The substrate for both phospholipase A$_1$ and lipoprotein lipase was an emulsion of glycerol trioleate and rat liver PC that was prepared by dilution of a clear mixture in glycerol (26).

For the phospholipase A$_1$ assays, 5 μCi of [1-^{14}C] PC was mixed with PC to give a total of 6.3 μmol and then dried under nitrogen at room temperature. Glycerol trioleate (133 μmol) and 2 ml of glycerol were added and the mixture was sonicated on ice for 1 min.

The substrate for triglyceride lipase was prepared in the same way except that it contained 10 μCi of glycerol tri-[1-^{14}C] oleate and 6.3 μmol of PC. Before use, the stock solutions were diluted with 4 parts (v/v) of 0.2 mol/l tris-HCl, pH 8.1, containing 30 g/l fatty acid free bovine serum albumin. The final concentrations of reagents in the medium were: PC, 0.52 μmol/ml; triolein, 11.1 μmol/ml; albumin, 20 mg/ml; glycerol, 4.5 mol/l; and tris-HCl, 0.133 mol/l.

Assay of Phospholipase A$_1$

Phospholipase A$_1$ activity was determined by the formation of 2-[1-^{14}C] linoleyl lysophosphatidylcholine. Fifty or 100 μl of enzyme was added to 200 μl of substrate and incubated at 37 C for 30 min. Then 6 ml of chloroform-methanol (2:1, v/v) was added followed by 2 ml of water. The mixture was vortexed, then centrifuged. The upper phase was removed, transferred to a glass tube and dried under nitrogen at 40 C. The residue was dissolved in 100 μl of chloroform containing 0.1 mg/ml lysophosphatidylcholine and then 50 μl was applied to a 20 x 20 cm sheet of glass fiber-Silica Gel G. The sheet was developed for 15 cm in chloroform-methanol-ammonia (80:30:2, v/v/v). The lysophosphatidylcholine zone was located with iodine vapor, cut out and transferred to a scintillation vial for counting. Results are means ± SEM (n = 6).

Assay of Lipoprotein Lipase

Lipoprotein lipase was determined by the

formation of [1-^{14}C] oleate using assay conditions similar to those described by Nilsson-Ehle and Schotz (26). Fifty or 100 μl of enzyme was added to 200 μl of substrate and incubated at 37 C for 30 min. then 3.25 ml of chloroform-methanol-heptane (1:1.25:1, v/v/v) was added followed by 1.05 ml of 0.1 mol/l potassium carbonate. The mixture was vortexed and centrifuged for 10 min at 1000 g and 1 ml of the upper phase was removed for counting.

Scintillation Counting

^{14}C Radioactivity was counted in a Packard Tri Carb scintillation spectrometer using 10 ml toluene-triton X 100, (2:1, v/v), containing 3.3 g/l PPO and 0.33 g/l POPOP as scintillators.

RESULTS

In most experiments, a mixed emulsion of PC and triolein was used that was stable for several hours at 37 C. Addition of lipoprotein lipase (final concentration 66 mu/ml) to the emulsion resulted in the degradation of both PC and triolein. Over 30 min incubation, the rate of lysophosphatidylcholine formation was 0.28 ± 0.01 nmol/min/ml (mean ± SEM, n = 6).

When serum was added to the substrate to a concentration of 1.5% v/v, conditions known to result in the activation of triolein hydrolysis by lipoprotein lipase (26), there was an increase in the rate of hydrolysis of both PC and triolein. For a 30 min incubation, the rate of lysophosphatidylcholine formation rose to 1.19 ± 0.02 nmol/min/ml and oleate formation rose to 64.4 ± 0.4 nmol/min/ml. The degradation of both PC and triolein in the mixed emulsion followed a similar time course (Fig. 1).

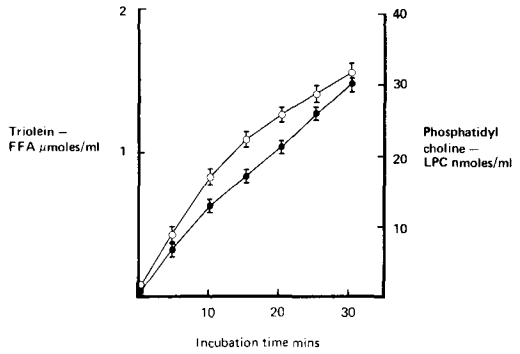

FIG. 1. Hydrolysis of PC- and triolein by lipoprotein lipase. Aliquots of enzyme (100 μl) were incubated at 37 C with 200 μl of a mixed emulsion of PC (0.52 mmol/l) and triolein (11.1 mol/l) containing either ^{14}C PC or ^{14}C triolein and added serum (1.5% v/v). Aliquots were removed at intervals for lysolecithin formation (•) or oleate formation (○).

Soluble apoproteins prepared by suspension of apo VLDL in 0.2 M tris-HCl buffer (pH 8.2) stimulated both the phospholipase A_1 and the triglyceride lipase activity when added to the substrate at concentrations up to 12.5 μg/ml (Table I). The activation of triolein degradation produced by the addition of increasing quantities of apo VLDL to the substrate paralleled the activation of PC degradation.

Individual soluble apoproteins prepared from VLDL—apo C-I, arginine-rich, apo C-II and apo C-III-1—were examined for activation of PC hydrolysis by lipoprotein lipase. Apoprotein C-II was the only apoprotein that produced a significant stimulation of PC degradation (Table

TABLE I

Effect of Apoproteins of Human VLDL on Phosphatidylcholine (PC) and Triolein Hydrolysis by Lipoprotein Lipase[a]

Added apoprotein	Phosphatidylcholine hydrolysis (nmol/min/ml)	% Control	Triacylglycerol hydrolysis (nmol/min/ml)	% Control
Control	0.32 ± 0.02	100	13.0 ± 0.5	100
Apo-VLDL	1.40 ± 0.02	437	65.1 ± 0.7	501
Apo C-I	0.35 ± 0.01	109	11.9 ± 0.6	99
Apo C-II	1.84 ± 0.01	575	62.4 ± 0.7	480
Apo C-III	0.34 ± 0.01	106	14.6 ± 0.5	112
Arg. rich	0.41 ± 0.02	128	14.1 ± 0.1	108

[a]Apo VLDL at a final concentration of 12.5 μg/ml and individual apoproteins isolated from apo VLDL at a final concentration of 5 μg/ml were added to substrate containing ^{14}C PC or ^{14}C triolein. Enzyme (100 μl) was incubated with 200 μl of substrate for 30 min at 37 C and lysolecithin or oleate formation was determined (see Methods). Results are mean ± S.E.M. of six assays.

I). As expected, apo C-II also produced a marked activation of triolein degradation.

Addition of apoprotein C-II to the substrate at a concentration of 5 μg/ml produced a four-fold increase in PC hydrolysis that was similar to the degree of activation achieved with apo VLDL. The activation of phospholipase A_1 activity paralleled the activation of triglyceride lipase activity (Fig. 2).

PC degradation by lipoprotein lipase and the activation of phospholipase A_1 activity were not restricted to PC-coated triolein particles. When micellar dispersions of 0.52 mmol/l PC were used as substrate, the rate of degradation of PC (0.37 ± 0.12 nmol/min/ml) was similar to that in mixed emulsions of 0.52 mmol/l PC and 11.1 mol/l triolein (0.43 ± 0.17 nmol/min/ml). Similarly, the activation of PC degradation by apo C-II occurred to the same degree in either PC dispersions, where it increased to 1.63 ± 0.19 nmol/min/ml after the addition of C-II to 5 μg/ml, or in mixed emulsions, where it increased to 1.71 ± 0.36 nmol/min/ml.

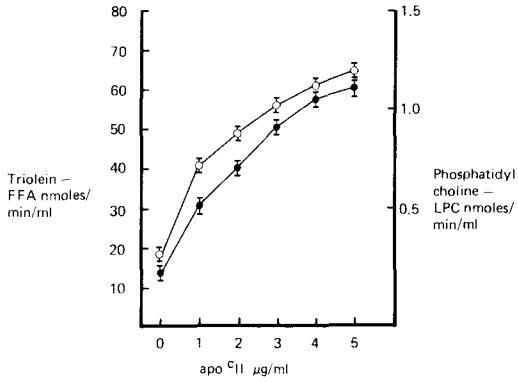

FIG. 2. Effect of VLDL apo C-II on triolein and PC hydrolysis. Apoprotein C-II was isolated from apo VLDL by gel filtration and DEAE-cellulose chromatography (see Methods). The apoprotein was added to the substrate and activation of phospholipase A_1 activity (•) and triacylglycerol lipase activity (○) was determined by lysolecithin or oleate formation after 30 min at 37 C.

DISCUSSION

The activation of lipoprotein lipase by apoprotein C-II is essential for maximal hydrolysis of triacylglycerols containing long chain fatty acids (9-14). This peptide is a constituent of the natural substrates of lipoprotein lipase, i.e., chylomicrons and VLDL, and suggests activation of lipoprotein lipase by apoprotein C-II is of physiological importance in the catabolism of these triacylglycerol-rich lipoproteins. This suggestion is supported by the demonstrated absence of C-II in one individual, resulting in a marked hypertriglyceridemia caused by defective clearance of triacylglycerol-rich lipoproteins from the circulation (27).

Previous studies have shown lipoprotein lipase has associated phospholipase A_1 activity degrading PC in lipoproteins (5,6), and this is confirmed by our observation that in emulsions of PC- and triolein-containing serum activator both components are degraded in parallel during incubation with a purified preparation of the enzyme. This contrasts with previous reports that lipoprotein lipase has little or no activity toward PC in synthetic substrates (18,28). The differences may result from the use of egg lecithin, which has a markedly different fatty acid composition to PC in plasma lipoproteins, or alternatively, to the lack of an activator of lipoprotein lipase in the substrate.

The rate of triolein hydrolysis was greater than PC hydrolysis (3). However, the relative rates of hydrolysis must be interpreted by observing that they may depend on relative substrate concentrations and by considering the chain length and degree of unsaturation of the PC fatty acids, as well as the physical state of the emulsion. In this study, the 1-acyl fatty acid in the labeled PC was primarily stearic acid, which is a major fatty acid component of VLDL PC (29), and the relative proportion of PC to triolein in the substrate was similar to the proportions in chylomicra (26).

Removal of PC during VLDL and chylomicron metabolism may be necessary for the maintenance of the appropriate ratio of polar surface components to apolar core components during degradation of core triacylglycerol by lipoprotein lipase (30). Although loss of phospholipid from these particles can occur by transfer to HDL (31,32), the observation that lipoprotein lipase can degrade PC at greatly increased rates in the presence of C-II activator supports the view that hydrolysis of PC is an important factor in the catabolism of triglyceride-rich lipoproteins. While C-II activation of lipoprotein lipase may be a requirement for PC hydrolysis, other factors may limit phospholipid degradation by the enzyme since PC of HDL is not degraded by lipoprotein lipase, even though the lipoprotein contains apo C-II.

Although the activating property of apo C-II has been localized to specific regions of its primary structure (33), the precise mechanism of activation is unknown. It may act by increasing enzyme substrate affinity (34). Since in mixed emulsions of PC and triolein the activation of triacylglycerol lipase and phospho-

lipase A_1 activity by C-II occurs in parallel, it is likely that a similar mechanism of activation is involved.

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METHODS

Analysis of Dolichol in Human Tissues by High Pressure Liquid Chromatography

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ABSTRACT

Dolichol from human liver was shown by reverse-phase high pressure liquid chromatography to consist of a series of homologues ranging in length from 17 to 23 isoprene units. The two major components, corresponding to 19 and 20 isoprene units, respectively, were isolated and identified by mass spectrometry. Dolichyl palmitate, synthesized from liver dolichol, showed an identical series of peaks with longer retention times. Attempts to chromatograph dolichyl phosphate under similar conditions were unsuccessful. Dolichol from uterine tissue and several other human tissues showed a shift toward shorter chain length, with a predominance of homologues containing 18 and 19 isoprene units.

INTRODUCTION

Dolichyl phosphate has been shown to act as an intermediate in glycoprotein biosynthesis (1-3), and this has stimulated interest in the distribution and metabolism of dolichol and its derivatives in mammalian tissues. Earlier studies in our laboratory showed that human tissues such as liver and endocrine glands were particularly rich in dolichol (4). The dolichol was present mainly as the free alcohol, but up to 20% was esterified with fatty acids in some tissues.

For these studies, the dolichol was isolated by column chromatography and quantitated by weighing the isolated material, but this method was time consuming and unsuitable for analysis of small quantities of dolichol. An isotopic method (5) was also found to be unsatisfactory for this purpose. Other workers have reported the use of high pressure liquid chromatography (HPLC) for analysis of dolichol, either as the free alcohol (6) or the *p*-nitrobenzyl derivative (7). Our experience has shown that HPLC of the free alcohol, using a simple solvent system and detection by UV absorption at 210 nm, provides a simple, rapid method for quantitating dolichol in small samples of tissue and determining the patterm of homologues. The same system can be used for analysis of fatty acid esters of dolichol.

MATERIALS AND METHODS

Preparation of Dolichol and Derivatives

Dolichol was isolated by column chromatography, as described previously, from human livers obtained at autopsy (4). Its identity was checked by thin layer chromatography (TLC) and by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy (8). This material was used to synthesize dolichyl palmitate by a method analogous to that used to synthesize cholesteryl esters (9). It was used also for synthesis of dolichyl phosphate (10) and as reference material for comparison with dolichol extracted from other human tissues obtained at surgery.

The unsaponifiable lipid from small samples (approximately 2 g) of these tissues was isolated by refluxing overnight in 5 ml of aqueous ethanolic KOH containing pyrogallol (11), extracting the digest 3 times with equal volumes of petroleum ether and washing the combined extract with water until neutral. The extract was concentrated on a rotary evaporator to about 4 ml, and a solution of 10% digitonin in 50% aqueous ethanol was added to precipitate most of the cholesterol. The precipitate was separated by centrifugation, washed with petroleum ether and the washings added to the supernatant. The resultant extracts were prepared for HPLC by evaporating the solvent and redissolving the residue in 2 ml of UV grade hexane or isopropanol.

Analysis by HPLC

Chromatography was performed on a Hewlett-Packard 1084A dual pump instrument equipped with a C18 reverse-phase column (Brownlee Labs, Santa Clara, CA). Eluates were monitored at 210 nm with a Hewlett-Packard 1030B variable-wavelength detector. The mobile phase was UV grade methanol/isopro-

panol (1:1). The column temperature was 55 C and the flow rate was 2 ml/min.

Linearity of response to dolichol was checked by chromatographing 10 μl of dolichol standard solutions ranging in concentration from 4 μg/ml to 4 mg/ml. Linearity was maintained within this range when the total area of all homologues was plotted against concentration. The minimum detectable amount of dolichol was 40 ng.

Mass Spectrometry of Homologues of Dolichol

Dolichol from human liver was injected repeatedly into the high pressure liquid chromatograph and fractions containing the two major homologues were collected until a sufficient amount was obtained for analysis by mass spectrometry. The homogeneity of the isolated peaks was confirmed by reanalyzing them individually by HPLC. Mass spectrometry of the separated homologues was performed on a Varian MAT-311A under conditions described previously (8).

RESULTS AND DISCUSSION

Separation of the homologues of dolichol and dolichyl palmitate by HPLC is illustrated in Figure 1. The palmitate ester was prepared from the dolichol isolated from human liver and showed an identical pattern of homologues. Attempts to chromatograph dolichyl phosphate on this column were unsuccessful. As expected for a reverse-phase system, the free alcohols, being more polar, have shorter retention times than the palmitate esters. In each case, the separation of the individual homologues probably also results from the greater polarity of the shorter chain components of the mixture.

The two major homologues of dolichol were isolated preparatively by HPLC and were shown by mass spectrometry to contain 19 isoprene units (mass ion 1312^{+}) and 20 isoprene units (mass ion 1380^{+}), respectively. On this basis, the other peaks were presumed to correspond to homologues containing 17-23 isoprene units.

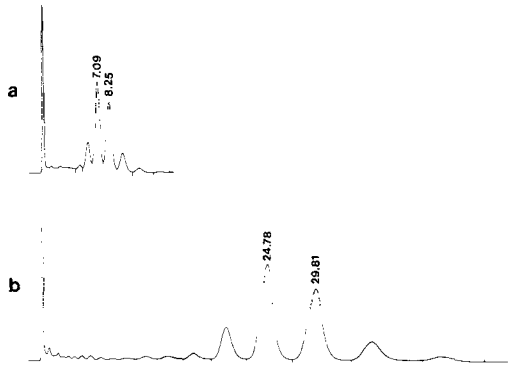

FIG. 1. Separation by HPLC of the homologues of (a) dolichol from human liver, and (b) dolichyl palmitate prepared from the liver dolichol. (See Materials and Methods section for conditions). The figures above the major peaks represent retention times. The initial peak results from the hexane in which the samples were dissolved for injection.

The relative proportions of these different homologues in human liver are given in Table I. A somewhat different pattern was observed in fresh human uterine tissue obtained at surgery (Table I). Analysis of single samples from pancreas and thyroid gave patterns similar to that of uterus, whereas a sample of testicular tissue showed a pattern similar to that of liver. The thyroid tissue analyzed in our studies was hyperactive from Grave's disease and the testicular tissue showed atrophy of the germinal epithelium. Pathological or postmortem changes may have influenced the results of

TABLE I

Homologues of Dolichol in Human Liver and Uterus[a]

No. of isoprene units	Retention time (min)	Liver[b] (n=6)	Uterus[b] (n=5)
17	5.29	0.9 ± 0.22	3.5 ± 0.40
18	6.10	8.8 ± 0.42	28.4 ± 1.10
19	7.08	36.6 ± 1.21	52.6 ± 0.41
20	8.25	37.3 ± 0.52	14.0 ± 0.92
21	9.66	12.4 ± 0.65	Trace
22	11.35	3.2 ± 0.26	
23	13.41	0.7 ± 0.12	

[a]Results are expressed as percentage of total dolichol ± SEM.
[b]n = number of samples analyzed.

some of our analyses, but the patterns for human liver and uterus were quite reproducible in tissues from different individuals (Table I). The different patterns may be an indication that tissues synthesize their own dolichol.

There is also some evidence that the pattern of homologues in a particular organ or tissue varies in different species. Tavares et al. (6) reported that pig liver dolichol consists of a mixture of homologues containing 17-21 isoprene units, with dolichol-19 predominating. Studies in our laboratory have shown rat liver dolichol is composed of homologues 16-21, with 18 and 19 predominating (unpublished experiments).

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LETTER TO THE EDITOR

A Note on the Spectrophotometric Determination of Long Chain Bases in Lipids

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We have recently reported (1) on a method for the quantitative determination of glycosphingolipids based on the spectrophotometric assay of a complex formed between sphingosine and methyl orange (2). For this purpose, the acetylated glycosphingolipid fraction obtained by column chromatography on Florisil was deacetylated and complexed with methyl orange. For calibration, a standard solution of sphingosine sulfate in chloroform/methanol/water (1:2:1, v/v/v) was used. No other long chain bases were measured.

It is known (3) that various homologs and analogs of sphingosine occur as constituents of glycosphingolipids. Hence, it is of considerable interest to know whether the molar absorption coefficients of the methyl orange complexes of various long chain bases are different. Some long chain bases prevailing in natural sphingolipids are available (Koch-Light Colnbrook, Buckinghamshire, England, or Sigma, St. Louis, MO), as such or as sulfates, and were shown to be chromatographically pure (4). It is evident from Table I that molar absorption coefficients of the various long chain bases studied are essentially identical (mean value 2.80×10^4), provided the amino group of the base is free.

Ceramide was isolated from bovine spleen lipids by the column chromatographic procedure of Vance and Sweeley, adapted for this study (5). Obviously, ceramide does not form a complex with methyl orange and only minimal absorption was detected at 415 nm. After hydrolysis (1), however, the sample complexed with methyl orange and an absorption of expected magnitude was observed. Thin layer chromatography (TLC) (4) revealed dihydrosphingosine to be the major component of the long chain bases of bovine spleen ceramide.

Peracetylation (*O*- and *N*-acetylation) of *erythro*-dihydrosphingosine with acetic anhydride in pyridine (6) yielded a ninhydrin-

TABLE I

Results of Assays of Various Long Chain Bases

Sample	Molar absorption coefficient $\epsilon_{415\,nm}$ $l \cdot mol^{-1} \cdot cm^{-1}$
Sphingosine (4*t*-Sphingenine)	2.83×10^4
erythro-Dihydrosphingosine (*erythro*-Sphinganine; DL-*erythro*-1,3-dihydroxy-2-amino-octadecane)	2.76×10^4
threo-Dihydrosphingosine	2.88×10^4
Phytosphingosine (D-ribo-1,3,4-trihydroxy-2-amino-octadecane)	2.73×10^4
Ceramide[a]	$\sim 0.01 \times 10^4$
N-acetyl-*erythro*-dihydrosphingosine[b] (containing traces of ninhydrin-positive material)	0.17×10^4
N-Acetyl-*erythro*-dihydrosphingosine[b], hydrolyzed (1)	2.72×10^4
erythro-Dihydrosphingosine, peracetylated[c]	0.12×10^4

[a]Isolated from bovine spleen.

[b]Prepared from *erythro*-dihydrosphingosine with acetic anhydride in methanol.

[c]Prepared from *erythro*-dihydrosphingosine with acetic anhydride in pyridine.

negative product that showed no absorption after attempted complexing with methyl orange. *Erythro*-dihydrosphingosine *N*-acetylated with acetic anhydride in methanol (7) also showed no absorption. The trace of absorption observed (Table I) probably results from residual unreacted based as revealed by TLC (4). After hydrolysis (1), both samples could be complexed with methyl orange, resulting in an absorption of the expected level.

These results show that the spectrophotometric determination of long chain bases of lipids (1) depends on the free amino group and (2) gives identical results with long chain base analogs.

ACKNOWLEDGMENT

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COMMUNICATIONS

Secondary Regulatory Sites in Rat Liver Cholesterol Biosynthesis: Role of 5-Pyrophosphomevalonate Decarboxylase

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ABSTRACT

The activity of 5-pyrophosphomevalonate decarboxylase in 43,000 g supernatant fractions from livers and kidneys of male adult rats has been determined. Enzyme activity in liver is significantly increased when rats are fed a diet containing 3% cholestyramine (268% of control rats) and decreased when fed a diet containing 2% cholesterol (25% of control rats). No circadian rhythm of enzyme activity is found in liver or kidneys. These results show that variations in hepatic cholesterogenesis affect the activity of 5-pyrophosphomevalonate decarboxylase in a similar way as other enzymes involved in the biosynthesis of cholesterol.

INTRODUCTION

The primary site for the regulation of cholesterol biosynthesis is at the step catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase which yields mevalonic acid (MEV) (1). Secondary sites for the regulation of this biosynthesis have also been proposed, both before (2) and after (3,4) the step catalyzed by the reductase. Ramachandran and Shah have suggested that the decarboxylation of 5-pyrophosphomevalonate (MEVPP) is a regulatory step, both in liver of suckling rats (5) and in developing rat brain (6). The results using suckling rats, although obtained by an indirect enzyme assay based on estimations of the amount of 5-phosphomevalonate (MEVP) and MEVPP formed and $^{14}CO_2$ liberated from [1-^{14}C]-MEV, indicated that cholesterol present in maternal milk would cause a decrease in activity of MEVPP decarboxylase (EC 4.1.1.33) while not affecting the preceding kinases.

A communication has recently appeared (7) showing that in adult rat liver homogenates there is both rhythmic activity in the utilization of [2-^{14}C]-MEV for cholesterol biosynthesis and in the liberation of $^{14}CO_2$ from [1-^{14}C]-MEV. These results, however, do not agree with a previous publication of Slakey et al. (4), who performed direct measurements of all the enzymes of the pathway of cholesterol biosynthesis between MEV and squalene in rat liver, and could not find significant diurnal variation in any of them.

These conflicting results, and the suggestion that the decarboxylation of MEVPP is a rate-limiting step in the synthesis of cholesterol in suckling rats and developing rat brain (4,6), and also in the synthesis of isoprenoids in *Chlorella* (8), prompted us to determine the activity of adult rat liver and kidney MEVPP decarboxylase, both at several times of the day, and under different dietary conditions.

MATERIALS AND METHODS

[2-^{14}C]-MEV (dibenzoylethylenediamine salt), 2,5-diphenyloxazole (PPO), and 1,4-bis-(2-(5-phenyloxazolyl))benzene (POPOP) were obtained from Amersham-Searle, England; bovine intestine alkaline phosphatase (Type I), rabbit muscle pyruvate kinase, ATP, DL-MEV lactone, phosphoenolpyruvate and 2-mercaptoethanol from Sigma Chemical Co., St. Louis, MO; cholesterol from Merck; and cholestyramine (Cuemid™) from Merck Sharp and Dohme, USA. All other chemicals were of analytical grade. [2-^{14}C]-MEVPP was synthesized enzymatically using partially purified MEV kinase and MEVP kinase from hog liver. A mixture of these enzymes was obtained by ammonium sulphate fractionation (30-60% saturation) and DEAE-cellulose column chromatography (Bazaes, S., personal communication). The incubation mixture contained 10 mM KF, 95 mM Tris-HCl pH 8.2, 3.8 mM $MgCl_2$, 6.2 mM phosphoenolpyruvate, 2 mM ATP, 38 mM KCl, 9 mM 2-mercaptoethanol, 4.5 mM [2-^{14}C]-MEV (2.45 x 10^5 cpm/umol), 800 units of pyruvate kinase, 44 units of MEV kinase and 104 units of MEVP kinase, all in a total volume of 440 ml, at 30 C for 90 min. The mixture was then acidified to pH 6 with N HCl, and the reaction was stopped by adding ethanol to 75% (v/v). Then it was warmed to 60

C for 1.5 min, cooled in ice, filtered, and the ethanol removed from the filtrate in a rotatory evaporator. The product was purified according to Dugan et al. (9). The final yield of the product was 60%, considering that only the (R)-isomer of the mixture of (RS)-MEV was used by the MEV kinase. The preparation contained no impurities as judged by paper chromatography and by spectrophotometric measurements.

Male rats of Sprague-Dawley strain, bred in this laboratory, were used. The animals were kept in a room with a light period from 8:00 a.m. to 8:00 p.m. and were fed ground rat pellets and water ad libitum. The basal diet (supplied by Alimentos Balanceados, Santiago) contained ca. 20% protein, 3.5% fat, 6% fiber, 67% carbohydrate, and a mineral-vitamin mix. For the experiments to test the effect of different diets, cholesterol and cholestyramine were incorporated into the diet to 2% and 3%, respectively.

Animals were sacrificed by cervical dislocation. Livers and kidneys were removed, chilled on ice, and homogenized in 2 volumes of cold 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The homogenate was filtered through several layers of cheesecloth and glass wool and centrifuged at 43,000 g for 1 hr at 4 C. The supernatant, after filtration through glass wool to remove lipid material, was used as the enzyme source. Protein concentration was determined by the biuret method (10). Incubations were carried out for 15 min at 37 C in rubber-stoppered 15 ml centrifuge tubes in 0.6 ml final volume containing 5 mM ATP, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 mM sodium acetate buffer, pH 4.9, 0.68 mM [2-^{14}C]-MEVPP (2.45 x 10^5 cpm/μmol) and 0.4-0.6 mg of protein. The reaction was stopped by the addition of 5.5 units of bovine intestine alkaline phosphatase in 1 M Tris-HCl buffer, pH 8.4, and further incubated for 2 hr at 37 C. Then, the incubation mixture was extracted two times with 1.0 ml of petroleum ether (bp 40-60 C). Radioactivity was measured in 1.0 ml of the combined hexane phases by adding it to 3.0 ml of a scintillation fluid containing 0.125 g POPOP and 4 g PPO in 1.1 liter of toluene/ethanol (10:1, v/v), and counted by conventional liquid scintillation spectrometry. The unit of enzyme activity is expressed in nmole of alcohols liberated per min, according to (4).

RESULTS

Preliminary experiments to establish optimal conditions for the assay of MEVPP decarboxylase in the 43,000 g supernatant fraction of rat liver revealed that the enzymic reaction is linear up to 25 min and proportional to the amount of added protein up to 0.8 mg. The optimum pH is between 4.0-4.7 in acetate buffer and apparent Km for MEVPP and ATP are 9.3 x 10^{-5} M and 1.7 x 10^{-4} M, respectively.

The effect of supplementing the diet of the animals with 2% cholesterol or 3% cholestyramine can be seen in Table I. Cholesterol reduces the enzyme activity 3.9 times while the effect of the bile acids-sequestering agent cholestyramine (11) is to increase its activity 2.7 times in liver. Both values are significant ($P < 0.002$) with respect to the control values. No significant effect is observed in the enzyme activity of the 43,000 g supernatant fraction from kidneys (results not shown).

Figure 1 shows the absence of rhythmic activity in MEVPP decarboxylase from rat liver and kidneys.

TABLE I

Animal Weights, Liver Weights, Protein Contents and Activity of MEVPP Decarboxylase in 43,000 g Supernatant Fractions of Liver of Rats under Different Diets[a]

Diet	Animals weights (g)	Liver weights (g)	Protein in 43,000 g supernatant (mg/liver)	Enzyme activity (nmoles/min/g liver)
Control	188 ± 9[b] (5)[c]	8.37 ± 0.47	242 ± 12	22.9 ± 1.5
2% cholesterol	209 ± 4 (5)	9.55 ± 0.47	271 ± 15	3.8 ± 0.8
3% cholestyramine	163 ± 10 (6)	6.88 ± 0.36	258 ± 19	61.5 ± 7.5

[a]Enzyme activity was determined in the 43,000 g supernatant fractions as described in Materials and Methods. Male animals were fed the different diets and water ad libitum for 6 days before the experiment, and were killed at 9:00 a.m.

[b] Mean ± S.E.M.

[c]Numbers in parentheses indicate the number of rats in each group.

DISCUSSION

It is known that the feeding of cholesterol or cholestyramine to rats has opposite effects on the rate of hepatic cholesterogenesis (1). The feeding of a diet containing 2% cholesterol to adult male rats decreases the activity of cytosolic acetoacetyl coenzyme A thiolase and cytosolic 3-hydroxy-3-methylglutaryl coenzyme A synthase to 29% and 15%, respectively, in 7 days (12), and of MEVPP decarboxylase to 25% in 6 days (Table I). When 3% cholestyramine is incorporated to the food of the animals, the values for the thiolase and synthase are increased up to 173% and 264%, respectively in 3 days (12), and the decarboxylase to 268% in 6 days (Table I). The effect of a 48 hr fasting, on the other hand, shows that cytosolic acetoacetyl coenzyme A thiolase, cytosolic 3-hydroxy-3-methylglutaryl coenzyme A synthase (12), MEVPP decarboxylase, isopentenylpyrophosphate isomerase, dimethylallyltransferase, and squalene synthetase (4) adapt to this new metabolic situation with a 2- to 7-fold decrease in activity. These findings suggest that the secondary control of cholesterol biosynthesis (1) is not due to an enzyme in particular but to groups of enzymes responding coordinately to the need for the synthesis of more or less cholesterol. It is very likely, then, that the activities of the enzymes catalyzing steps between isopentenylpyrophosphate and squalene should also present coordinate variation in the liver of cholesterol- or cholestyramine-fed rats. As has been demonstrated by several workers, the response of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase to cholesterol/cholestyramine feeding or fasting is rapid enough to account for primary control of cholesterogenesis in the rat (1).

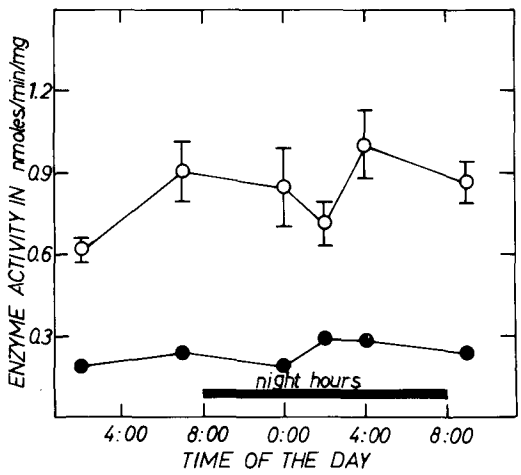

FIG. 1. Absence of circadian rhythm of MEVPP decarboxylase from rat liver and kidneys. Enzyme activity was determined in 43,000 g supernatant fractions from liver and kidneys as described in Materials and Methods. Activity for liver MEVPP decarboxylase (0) is given as the mean ± S.E.M. (5 animals). The activity of MEVPP decarboxylase from kidneys (•) was determined in 43,000 g supernatant fractions from pooled kidneys obtained at the different times.

Recently, Shama Bhat and Ramasarma (7) have suggested that one or more of the three enzymes between MEV and isopentenylpyrophosphate present circadian rhythm in rat liver. However, since neither the activity of MEV kinase or MEVP kinase decline in the liver of fasting rats, while the level of MEVPP decarboxylase presents a 2-fold fall in activity (4), it seemed to us more probable that the enzyme responsible for such rhythm (7) would be the decarboxylase rather than any or both of the preceeding kinases. Our results, however, indicate that MEVPP decarboxylase does not present diurnal variation in activity (Fig. 1), so that the rhythm observed by Shama Bhat and Ramasarma (7) should be due to variations in one or both kinase. It must be emphasized, however, that several reports from different laboratories have failed to demonstrate diurnal rhythm for the incorporation of [2-^{14}C]-MEV into cholesterol in rat liver (11), for any of the enzymes-catalyzing steps between MEV and aqualene in the same organ (4), or for rat adrenal glands MEV kinase (13). Perhaps some uncontrolled factor present in the rats or the assay used by those authors (7) is responsible for their results. It is interesting to note, in this regard, that when we assayed the activity of MEVPP decarboxylase in the 800 g supernatant, as determined by those authors (7), the specific activity of the decarboxylase was 47% and the total units of enzyme activity were 69% of those found in the 43,000 g supernatant fraction of rat liver (Jabalquinto, A.M. and E. Cardemil, unpublished results).

Our results on the absence of response of MEVPP decarboxylase from rat kidneys to cholesterol or cholestyramine feeding, and the absence of diurnal variation in activity (Fig. 1) constitute, to the best of our knowledge, the first direct determinations of this enzyme activity in that organ. The lack of diurnal variation is in keeping with our result in liver, and the absence of response to dietary conditions may indicate that the secondary control of cholesterol biosynthesis in kidneys (if any) is probably different to that of liver. More experiments are needed, however, before definitive conclusions can be drawn in this respect, especially in view of the importance of the

kidneys in the metabolism of MEV via the shunt pathway (1,14).

After this paper was submitted for publication, a recent report from Shama Bhat and Ramasarma (15) came to our attention in which a 2-fold increase of MEVPP decarboxylase activity was observed in a 12 hr period of time in rat liver.

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5,9,23-Triacontatrienoic Acid, Principal Fatty Acid of the Marine Sponge *Chondrilla nucula*

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ABSTRACT

The marine sponge *Chondrilla nucula* contains 34% 30:3Δ5,9,23 in its total fatty acids. This non-methylene-interrupted polyunsaturated acid, unknown in other living organisms, occurs mainly as an ester in phosphatidylethanolamine.

INTRODUCTION

Recent studies in our laboratory have shown that marine sponges of the class Demospongiae contain high levels of C_{24}-C_{30} "demospongic" fatty acids possessing new and novel structures not found in other organisms. We have identified, for example, 26:2Δ5,9 and 26:3 Δ5,9,19 in *Microciona prolifera* (1), 26:2Δ5,9 and 28:3Δ5,9,19 in *Xestospongia halichondroides* (2), and 30:4ω6 and 30:5ω3 in *Cliona celata* (3).

Our initial survey of fatty acid chain lengths in sponges (4) had revealed that the chicken liver sponge, *Chondrilla nucula*, contained 38% C_{30} chains in its total fatty acids. We have now characterized the structure of this C_{30} component and have identified it as a new polyunsaturated fatty acid.

EXPERIMENTAL PROCEDURES

C. nucula sponge colonies collected near Little Torch Key, Florida were purchased from Marine Specimens Unlimited (Summerland Key, Florida) in July 1977 and August 1978. The 1977 sample was shipped by air to our laboratory. It was used only for fatty acid structure experiments, since an appreciable amount of free fatty acid was found in the lipid extract, probably produced by hydrolytic degradation in transit. The 1978 sample was extracted in Florida immediately after collection. Lipids from this sample showed negligible free fatty acid content and were used for lipid class studies.

Sponge colonies were washed in seawater, carefully cleaned of all nonsponge debris, and cut into small pieces. Immediate extraction with chloroform/methanol (first 1:2, then 2:1) yielded the total lipids (2.5% of dry weight). BHT was added to this sample and to all further lipid isolates to retard autoxidation.

Methyl esters were prepared from total *Chondrilla* lipids by refluxing in methanol-conc. H_2SO_4, 98:2, for 3 hr. They were purified by preparative thin layer chromatography (TLC) on 1.0 mm thick silicic acid developed in hexane/diethyl ether, 96:4. The yield of methyl ester was ~23% of the total lipids reacted.

Our procedures for TLC, gas liquid chromatograph (GLC), mass spectrometry, UV spectroscopy, IR spectroscopy, proton magnetic resonance (PMR) analysis, hydrogenation, and ozonolysis of fatty acid methyl esters have been described in two previous publications (2,3). The pyrrolidide of 30:3 was prepared using the method of Andersson and Holman (5), purified by TLC (chloroform/diethyl ether, 90:10), and then analyzed on a Hewlett-Packard Model 5992A GLC mass spectrometer equipped with a 600 x 2.4 mm id, 2% OV-101 GLC column at 250 C. The free acid form of 30:3 was obtained from the purified ester by saponification with 10% KOH in methanol, purified by TLC (hexane/acetone, 85:15), and then recrystallized from acetone at -20 C.

Lipid class separations utilized column chromatography followed by preparative TLC. Total *Chondrilla* lipids (285 mg) were first separated on a 20 x 50 mm column of silicic acid (Silica Gel G50, E. Merck, Darmstadt, Germany) into four fractions by successive elution with 1.5 column volumes of $CHCl_3$, 2 volumes of acetone, 5 volumes of $CHCl_3$/CH_3OH (1:1), and 4 volumes of CH_3OH. The $CHCl_3$ fraction was further separated by preparative TLC on Whatman K5 silica gel plates (Whatman Inc., Clifton, NJ) developed in hexane/diethyl ether, 95:5. The sterol ester and triglyceride bands were visualized with rhodamine 6G; each was scraped off the plate and recovered with diethyl ether. The remaining adsorbent was also removed and eluted with diethyl ether; this eluate was added to the acetone column fraction to form the "other neutral lipids" sample.

The $CHCl_3$/CH_3OH column fraction was separated by TLC ($CHCl_3$/CH_3OH/NH_4OH, 65:32:3). A glass plate was placed over the center of the chromatogram, and the lipids were located by exposing the edges to iodine vapor. The phosphatidylethanolamine and

phosphatidylserine bands were each scraped off the center of the plate and recovered by extraction with $CHCl_3/CH_3OH/H_2O$, 45:45:10. Phosphatidylcholine was isolated from the CH_3OH column fraction in the same manner. All remaining adsorbent from both polar lipid separations was extracted with $CHCl_3/CH_3OH/H_2O$, 45:45:10 to recover the "other polar lipids" sample. The identities of the phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine samples were confirmed by checking R_f values against known standards in various TLC developing solvents and by Dragendorff and ninhydrin spray reagent tests (6).

The amount of 30:3Δ5,9,23 in each lipid class was determined by GLC with an internal standard. Methyl esters were prepared from each isolate (7) and then purified by TLC. These esters were divided into two equal aliquots; a known amount of methyl triacontanoate (30:0) was added to one of them; and then both were analyzed by GLC. Comparison of the 30:3 peak area with that of the 30:0 standard (corrected for any minor peaks having the same retention time) permitted calculation of the weight of 30:3 present in each lipid class.

RESULTS

Purification of Methyl Esters

GLC analysis of *C. nuclula* total methyl esters on a Silar-10C column at 200 C revealed a large unknown peak having an ECL value (8) of 32.04. This peak constituted 33.5 wt. % of the total methyl esters.

To purify this unknown for further study, we first separated *Chondrilla* total methyl esters by TLC into two bands. The upper band containing the ECL 32.04 ester was recovered and then further fractionated using Ag^+-TLC. Development with 80:20 hexane/diethyl ether resolved five distinct bands. The center band ($R_f \cong$ triene) was recovered, purified a second time by Ag^+-TLC, and then repurified by normal TLC. The final product proved 95.6% pure by GLC, with the major impurity being 1.8% of an ECL 31.45 methyl ester.

Identification of 30:3

Hydrogenation of the ECL 32.04 methyl ester produced an n-30:0 peak when characterized by GLC. Mass spectrometric analysis give a molecular ion at m/e 460. Thus, an n-30:3 structure was indicated.

The ester showed no absorption bands in the 220-320 nm region, proving the absence of conjugated double bonds (9). Its infrared spectrum had no prominent absorption in the 970 cm^{-1} region, so all double bonds were of *cis* configuration (10). Typical 3000-2800 cm^{-1} hydrocarbon and 1720 cm^{-1} ester bands were observed, and the absorption pattern in the 1500-1100 cm^{-1} region was the same as for 18:3Δ5,9,12 (11). The PMR spectrum corresponded to that of an unsaturated methyl ester. Integration of the CH_3O- and -HC=CH-signals gave a 5.8:3.0 ratio indicating a trienoic compound. However, there was no $=C-CH_2-C=$ signal at 2.7 δ, showing the absence of a methylene-interrupted double bond system (12).

Reductive ozonolysis of the 30:3 unknown produced four products. These were identified by GLC as a C_5 aldehyde-ester, a C_4 dialdehyde, a C_{14} dialdehyde, and a C_7 aldehyde using cochromatography with authentic standards (C_4-C_7 compounds) or an extrapolated ECL plot (C_{14} dialdehyde). Hence, the structure was either 30:3Δ5,9,23 or 30:3Δ5,19,23. Mass spectrometry of the 30:3 pyrrolidide derivative proved Δ5 and Δ9 unsaturateion based on the interpretation of Andersson et al. (13): C_4 (m/e 140) = 19%, C_5 (m/e 152 + 153 + 154) = 25%, C_6 (m/e 166 + 167 + 168) = 39%, C_7 (m/e 180) = 100%, C_8 (m/e 194) = 10%, C_9 (m/e 207 + 208) = 15%, C_{10} (m/e 220) = 11%, C_{11} (m/e 234) = 13%.

We conclude that the unknown compound was the methyl ester of *cis*-5,*cis*-9,*cis*-23-triacontatrienoic acid. Pure 30:3Δ5,9,23 fatty acid had a melting point of 26.0-26.8 C (uncorrected).

Location in *C. nucula* Lipids

To determine how the unusual 30:3Δ5,9,23 acid was distributed among the various lipids of the sponge, the five major classes of *Chondrilla* ester-lipids were isolated by consecutive column and thin layer chromatography. All the remaining components were combined into "other polar lipids" and "other neutral lipids" samples. Each sample was converted to methyl esters, which were analyzed by GLC with an internal standard to determine the weight of 30:3 Δ5,9,23 present.

Results (Table I) show that the 30:3Δ5,9,23 chains are mainly located in the phosphatidylethanolamine molecules. One-eighth of the 30:3 appears in the triglycerides, and another eighth is esterified in the phosphatidylserine. Only minor amounts (<0.06) are present in other lipid classes.

DISCUSSION

The 30:3Δ5,9,23 fatty acid identified here

TABLE I

Distribution of 30:3Δ5,9,23 in *Chondrilla nucula* Lipid Classes

Lipid class	30:3Δ5,9,23 Present
Phosphatidylethanolamine	0.65
Phosphatidylserine	0.11
Phosphatidylcholine	0.01
Other polar lipids	0.03
Triglyceride	0.13
Sterol ester	0.01
Other neutral lipids	0.06
Total lipids	1.00

in the marine sponge *C. nucula* has not, to our knowledge, been previously found in other living organisms. Schmitz and McDonald (14) have characterized *Chondrilla* cerebrosides but found only C_{16}-C_{26} α-hydroxy acids present. The hydrocarbon chain of 30:3Δ5,9,23 contains the typical Δ5,9-diene structure encountered in other polyunsaturated demospongic acids such as 24:2Δ5,9, 25:2Δ5,9, 26:2 Δ5,9, 26:3Δ5,9,19, 27:3Δ5,9,20 and 28:3 Δ5,9,19 (1,2,15). The 5,9,23-triacontatrienoic acid is the principal fatty acid (34-38%) of *Chondrilla;* other acids present are mainly C_{16} (25%), C_{18} (10%), or C_{20} (11%) chain lengths (4).

Morales and Litchfield (16) have shown that the sponge *M. 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 a similar biosynthetic pathway may operate in *Chondrilla* to produce 30:3Δ5,9,23 from palmitoleic acid:

$$16{:}1\Delta9 \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow 30{:}1\Delta23 \rightarrow 30{:}2\Delta9,23 \rightarrow 30{:}3\Delta5,9,23$$

C. nucula is the fourth marine sponge in which the unusual C_{24}-C_{30} demospongic acids are known to be major components of membrane-type phospholipids. Phosphatidylethanolamine contains large amount of 26:2 and 26:3 in *M. prolifera* (1) and *Halicondria panicea* (17) and most of the 30:3 in *C. nucula.* Phosphatidylserine contains high levels of 26:2 and 26:3 in *M. prolifera* (1) and large amounts of 30:4 and 30:5 in *C. celata* (3). These findings imply that demospongic acid chains may participate in the membrane lipid bilayers of sponges. Since 30:3Δ5,9,23 acid has a melting point (26.0-26.8 C) lower than palmitic (63 C) but higher than oleic (13 C) acid, one would not expect 30;3-containing membranes to be extremely rigid as previously proposed (18). However, the increased length of the C_{30} chains should produce bilayers of unusual bulk and structure (18).

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LETTER TO THE EDITOR

Antioxidants in Neoplastic Cells: III. An Antioxidant Artifact in Tissue Culture Media

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The membranes of mouse neuroblastoma cells exhibit a striking increase in resistance to lipid peroxidation during differentiation in tissue culture (1). High resistance to peroxidation is characteristic of many neoplasms (2), and comparison of undifferentiated (peroxidation-labile) neuroblastoma cells with differentiated (peroxidation-resistant) cells affords a useful experimental system for investigating the mechanisms underlying this resistance phenomenon. We recently presented evidence that differentiating neuroblastoma cells become resistant to peroxidation by generating A274, a phenolic antioxidant (3). We have now shown that the resistance phenomenon is not caused by the presence of A274. This antioxidant was found to be a contaminant widely distributed in tissue culture media.

In the studies reported here, mouse neuroblastoma cells (C1300, clone N18) were cultured and induced to differentiate by withdrawal of serum from the medium as previously described (1). Serum was removed from undifferentiated cells on the fourth day of subculture, and differentiated cells were harvested on the fifth day. The antioxidant activity of lipid samples was estimated by the sensitive, single-phase assay developed in this laboratory (1,3). The procedures used for lipid extraction, LH-20 Sephadex chromatography, protein determination, and lipid peroxide estimation have been reported previously (1,3).

The unusual two-line spectrum of A274 (3) provided a means of detecting this antioxidant in lipid extracts from neutroblastoma cells and commercial tissue culture medium. Lipid extracts were chromatographed on LH-20 Sephadex, and that fraction of the ethanol eluent previously found to contain A274 (1.0 V_{bed} to 1.5 V_{bed}) was evaporated, brought up in a small volume of chloroform and subjected to chemical ionization or electron impact mass spectrometry using a direct insertion probe. The instrumentation for mass spectrometry has been described (3). Based on our previous studies of A274 (3), a high ion current at m/z 181 was taken as evidence for the presence of this substance.

We have reported that A274 and its concomitant antioxidant activity increased dramatically during the differentiation of neuroblastoma cells (3). However, we recently observed the abrupt disappearance of A274 from our cell preparations. The fraction derived from LH-20 Sephadex chromatography which had contained A274 in previous experiments (3) yielded no peak of antioxidant activity, and mass spectra of this fraction did not contain ion currents above background noise at m/z 181. Since differentiated neuroblastoma cells remained highly resistant to lipid peroxidation, A274 cannot be related to the resistance phenomenon. A274 was shown to be absent from the lot of powdered tissue culture medium used in recent experiments, but this substance was detected in the lot of medium employed previously. Subsequently, we have observed that differentiated neuroblastoma cells contain A274 only when grown in lots of medium that contain A274. Since several lots of medium were used in our earlier studies, the consistency of our previous observations probably resulted from the wide distribution of A274 in commercial tissue culture medium. In a survey of media obtained from four suppliers, more than half the lots were contaminated with A274, and at least one sample from each supplier was contaminated.

A likely explanation for the large increase of A274 in neuroblastoma cells during differentiation is that the contaminant binds to serum proteins and is taken up by the cells in significant amounts only if serum is withdrawn from the medium. The binding of contaminants is one of several actions of serum in tissue culture (4). In light of the recent progress in minimizing the serum concentrations required

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for tissue culture (4), artifacts similar to those described in this report may be observed by other investigators.

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Gas Chromatographic Separations of Di- and Monoacylglycerols Based on the Degree of Unsaturation and Positional Placement of Acyl Groups

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ABSTRACT

Gas liquid chromatographic analysis of diacylglycerols (DGs) and monoacylglycerols (MGs) prepared by Grignard degradation of the triacylglycerols of plant and fish oils was examined as acetate and trimethylsilyl (TMS) ether derivatives on several polar cyanosiloxanes. Satisfactory separations of mixtures of DGs or MGs from simple plant oils based on carbon number and degree of unsaturation, as either acetates or TMS ethers, were obtained on SILAR 10C. In addition, the TMS ethers were effectively separated on the basis of the positional distribution of fatty acid in the acylglycerol molecule, but the acetates of the positional isomers overlapped. The equivalent chain lengths (ECLs) of DGs with 28-42 total acyl carbons and 0-6 double bonds, and MGs with 14-22 acyl carbons and 0-6 double bonds, are presented for both derivatives. The influence of column temperature on the ECLs is discussed.

INTRODUCTION

The development of the cyanosiloxane liquid phases such as SILAR 10C, which have high polarity and moderate thermal stability, has permitted gas liquid chromatography (GLC) of lipid components with higher molecular weights (MW) based on carbon number and degree of unsaturation, as indicated in our earlier work on wax esters (1,2), fatty acid steryl esters (3,4) and triacylglycerols (TGs) (5). This study deals with GLC separations of diacylglycerols (DGs) and monoacylglycerols (MGs) on these polar phases.

DGs and MGs have usually been submitted to GLC as acetyl or trimethylsilyl (TMS) derivatives. The GLC of the DG derivatives has been carried out almost exclusively on thermostable nonpolar liquid phases such as OV-1, JXR and SE-30 (6-8). The results have usually been presented in compositions based on the number of total acyl carbons only. Recently, Myher et al. (9,10) reported GLC resolution of DGs as TMS ethers based on carbon number, degree of unsaturation and positional distribution of fatty acid in the DG molecule on SILAR 5CP, and applied the method to the analysis of peanut oil TGs. We have now found that the use of SILAR 10C, which has a higher polarity than SILAR 5CP, gives notable advantages: much lower elution temp for both acetate and TMS ether derivatives, more stable baseline under both isothermal and temperature-programmed conditions up to 270 C, higher separation factors for homologs differing by one double bond and better separation for mixtures of positional isomers submitted as TMS ethers. These advantages have also been recognized for GLC of MG derivatives, which was carried out on SILAR 5CP and polyester columns previously (11,12). Dyer and Klopfenstein (13) used SILAR 10C in GLC analysis of the TMS ethers of DGs derived from the phosphatidylcholines (PCs) of rat liver. However, the main objective of their study was related to biochemical research on phospholipids, and a practical GLC technique was not presented. In this study, the GLC behavior of DGs and MGs (as either TMS ethers or acetates), on SILAR 10C is reported in detail.

MATERIALS AND METHODS

Di- and Monoacylglycerols

Synthetic 1,2- and 1,3-DGs of myristic, palmitic, stearic and oleic acids (P.L. Biochemiclas, Inc., Milwaukee, WI), and 1(3)-MGs of stearic and oleic acids (Tokyo Kasei Ltd., Tokyo, Japan) were used as references.

DG and MG samples were prepared by Grignard degradation (14) of the TGs of corn, safflower, linseed, sperm whale and Japanese anchovy oils with ethyl magnesium bromide. DGs and MGs in the degradation products were separated by thin layer chromatography (TLC) on Silica Gel G impregnated with 10% (w/w) boric acid. The plates were developed with chloroform/acetone (98:2, v/v) for DGs, and with chloroform/methanol (98:2, v/v) for MGs. Saturated DGs (C28-C42) and MGs (C14-C24) prepared from fully hydrogenated TGs of the anchovy oil were used for the determination of the equivalent chain lengths (ECLs) of unsaturated DGs and MGs, respectively.

The acetyl and TMS derivatives of DGs and MGs were prepared with pyridine/acetic anhydride (1:10, v/v) and pyridine/hexamethyl-

disilazane/trimethylchlorosilane (12:5:2, v/v), respectively (7).

Fractionation of DG acetates according to the degree of unsaturation was carried out by TLC on Silica Gel G impregnated with 15% (w/w) silver nitrate ($AgNO_3$-TLC). The plates were developed with benzene/chloroform (9:1, v/v) for the acetates from sperm whale oil, and with chloroform/methanol (99:1, v/v) for the acetates from safflower oil.

Gas Liquid Chromatography

GLC of DG and MG derivatives was carried out with GC-6AM and GC-7A Shimadzu gas chromatographs equipped with dual hydrogen flame ionization detectors. The columns were glass tubes (1.5 or 2 m in length, 3 mm id). The column packing materials, containing 3 or 5% (w/w) SILAR 10C, 5% SILAR 7CP, 5% SILAR 5CP and 5% OV-275 on 100-120 mesh Gas Chrom Q, were obtained from Applied Science Laboratories (State College, PA).

Isothermal analyses were carried out at various temp within the range 250-270 C for DG acetates and TMS ethers, 240-260 C for MG acetates, and 170-220 C for MG-TMS ethers. Temperature-programmed analyses were carried out under conditions of 200 to 270 C at 1 C/min in all cases, and the final temp was held until all peaks of acylglycerols emerged. The detector and on-column injector, in the same heater block, were maintained at 300 C for DG acetates, 270 C for DG-TMS ethers and MG acetates, and 240 C for MG-TMS ethers. The carrier gas was nitrogen. The sample size was usually 0.4-0.6 μl of 0.5% (w/v) solution of acylglycerols in hexane; attenuation was set in the region of 1 or 2 x 10^2.

GLC peaks were identified with reference standards, subfractions separated by $AgNO_3$-TLC, and fatty acid compositions. Peak area percentages and retention times (RTs) between the front of solvent deflection and the peak maximum were measured with a computerized digital integrator (Chromatopac ElA, Shimadzu).

RESULTS AND DISCUSSION

Column Efficiency of Several Cyanosiloxanes

Table I compares the number of theoretical plates (n), height equivalent to one theoretical plate (HETP) and RT of monooleoylglycerol and dimyristoylglycerol (as acetates and TMS ethers) in the GLC on several cyanosiloxane columns. The data show that the HETP and n of the MG and DG are not much different among the columns, but the RT significantly decreases with increasing polarity of the

TABLE I

Comparison of Column Efficiency[a]

Column	1(3)-Monooleoylglycerol						1,3-Dimyristoylglycerol					
	TMS ether			Acetate			TMS ether			Acetate		
	n	HETP	RT	n	HETP	RT	n	HETP	RT	n	HETP	RT
SILAR 5CP (5%, 1.5 m)	2152	0.70	62.3	2323	0.65	70.4	2468	0.61	118.2	2290	0.66	91.5
SILAR 7CP (5%, 1.5 m)	1530	0.98	29.6	1626	0.92	45.9	1905	0.78	49.7	1597	0.94	44.8
SILAR 10C (5%, 2 m)	1874	1.07	15.1	3098	0.65	30.7	2866	0.70	19.6	3243	0.62	19.8
SILAR 10C (3%, 1.5 m)	---	---	---	2187	0.69	17.9	---	---	---	2216	0.68	12.8
OV-275 (5%, 2 m)	1601	1.25	6.5	2588	0.77	11.2	1877	1.07	7.7	---	---	---

[a]n = number of theoretical plates; HETP = height equivalent to one theoretical plate (mm); RT = retention time from injection point to peak maximum (min); GLC conditions: column, glass tubes (3 mm id); column temp = 200 C for monooleoylglycerol TMS ether, 240 C for monooleoylglycerol acetate and dimyristoylglycerol TMS ether, and 270 C for dimyristoylglycerol acetate; sample size = 1 μl of a 0.5% solution in hexane; nitrogen flow rate = 13 ml/min.

siloxane liquid phases from SILAR 5 CP to OV-275. This influence of the liquid phase polarity on RT has already been observed in the GLC of wax esters, fatty acid steryl esters and TGs on polar and nonpolar columns; that is, the elution times of these compounds on SILAR 10C were much shorter than those on nonpolar columns such as OV-1 and JXR under the same conditions (1-5). Similar observations were obtained in this study on the GLC of DG and MG derivatives. These results can be explained by the low affinity of the nonpolar compounds to the polar phases. The 3% SILAR 10C column indicated slightly higher efficiency than that of the 5% SILAR 10C column for the acetates of DGs higher than C36. This improved efficiency on 3% SILAR 10C column has already been observed in the GLC of steryl esters (4) and TGs (5). However, the 3% SILAR 10C column showed considerable tailing for the TMS ethers of MGs and DGs. Thus, the 3% column was used only in the analysis of DG acetates in this study.

Resolution of Trimethylsilyl Ether and Acetate Derivatives on SILAR 10C

Figure 1 shows the resolution of the positional isomers of linseed oil MGs as TMS ethers. The 1(3)-isomers were clearly resolved from corresponding individual 2-isomers, but application was hindered by certain critical pairs which were observed between the isomer peaks (Fig. 1C). Effective resolution of the positional isomers was also obtained on SILAR 5CP, 7CP and OV-275, but the resolution between the isomer peaks was less satisfactory than that on SILAR 10C.

Figure 2 shows the resolution of corn oil DGs as TMS ethers. In comparison to SILAR 5CP and polyester columns (9,15), the SILAR 10C column showed a stable baseline without peak noise at high temp (270 C). Complete separations based on carbon number and degree of unsaturation were obtained for both 1,2(2,3)- and 1,3-DGs (Figs. 2A and 2B). The resolution between the isomer peaks (Fig. 2C) was much better than that already documented on SILAR 5CP; that is, the peaks 1,3-(18:1-18:1) and 1,2-(18:1-18:2), and 1,3-(18:1-18:2) and 1,2-(18:2-18:2) were not resolved on SILAR 5CP (9). To elute the positional isomers with satisfactory resolution, a carrier gas flow rate of less than 20 ml/min was required.

Resolution of the positional isomers of DGs and MGs (as acetates) on polar columns has not been reported. The cyanosiloxane columns used in this study also showed no resolution for the isomeric acetates. Figure 3 shows the resolution of safflower oil DGs (as acetates) on SILAR 10C. At 270 C, the DGs of C36 with 1-4 double bonds were eluted with complete resolution based on their degree of unsaturation. The corresponding TMS ethers also showed the same elution pattern at lower column temp (about 250 C). The 18:0-18:2 species is retained slightly longer than the 18:1-18:1 species (Figs. 2A and 3A). A similar observation was noted in the GLC of the TMS ethers on SILAR 5CP (9). The resolution of these species on SILAR 10C was somewhat improved at lower column temp but a clear-cut resolution was not obtained.

Figure 4 shows the resolution of sperm whale oil DGs as acetates. The DGs of C22-C40 with 0-2 double bonds were clearly resolved by temperature-programmed GLC. In the temperature-programmed analysis on SILAR 10C, a stable baseline was obtained more easily than with SILAR 5CP and 7CP. The partial resolution of carbon numbers 26, 28, 30 and 32 in monoenes (Fig. 4C), and 30, 32, 34 in dienes (Fig. 4D) probably results from the shifts of the DG isomers of the same MW but different fatty acids, such as the 18:1-18:1 and 18:0-18:2 species in Figure 3. The fatty acids of the monoene fraction were saturates of C12-C18 and monoenes of C14-C20, and those of the diene fraction were saturates of C14-C18, monoenes of C14-C20 and a diene of C18. These compositions suggest the peak 30:1, for example, consists mainly of the 12:0-18:1, 14:0-16:1 and 16:0-14:1 species, and the peak 32:2 consists mainly of the 16:1-16:1, 18:1-14:1 and 14:0-18:2 species.

Figure 5 shows the elution pattern of anchovy oil MGs (as acetates) obtained by temperature-programmed GLC. The chromatogram is characterized by a stable baseline, complete separations of various components and an elution order of 20:0, 18:2, 20:1, 18:3 and 22:1 species. The corresponding TMS ethers and methyl esters were eluted with some overlapped peaks, and the resolution of the saturated and unsaturated species was not as clear as for the acetates.

Equivalent Chain Lengths and Separation Factors

Tables II and III list the relative retention data for MGs and DGs on SILAR 10C, respectively. The ECL widely used for fatty acid methyl esters is based on the linear relationship between the log of RTs of normal saturated esters and their carbon numbers (16). In this study, however, the acetates and TMS ethers of saturated MGs and DGs showed slightly negative or positive deviations from straight lines under isothermal conditions. Therefore, the ECLs of unsaturated MGs and DGs were

calculated from the log of the relative retention times (RRTs) of neighboring saturated homologous pairs with even carbon chains. Nelson (17) reported the plot of the adjusted RTs against carbon number in fatty acids on capillary columns coated with diethylene glycol succinate was not linear but was best approximated by a second order equation. Table IV compares the carbon number calculated on the basis of a straight line and a parabola for saturated DGs with 28-42 total acyl carbons. The acetates, which show slightly positive deviation from a straight line, are approximated well by the following equation:

$$\log(RRT) = 0.0004875(CN)^2 + 0.02598(CN) - 1.3306,$$

where RRT is RT relative to dipalmitoylglycerol acetate at 270 C and CN is total acyl carbon number. Such a quadratic relationship, however, is not apparent for the TMS ethers, which show slightly positive deviation from a straight line. The acetates of saturated MGs showed slightly negative deviation only in higher carbon number homologs (C20-C24).

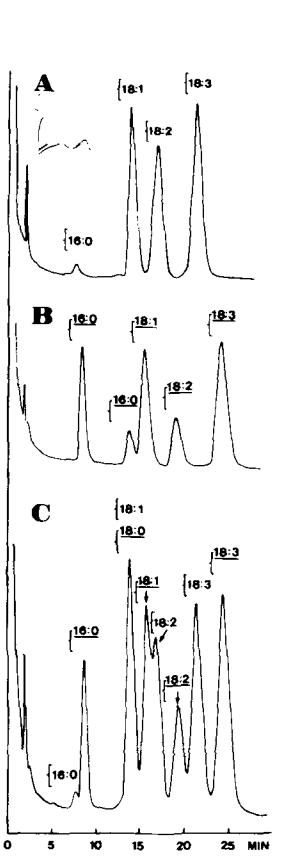

FIG. 1. Gas chromatographic resolution of linseed oil monoacylglycerols as TMS ethers on SILAR 10C. (A) 2-monoacylglycerols; (B) 1(3)-monoacylglycerols; (C) ca. 1:1 mixture of A and B. GLC conditions: column, glass tubes (2 m x 3 mm id) packed with 5% SILAR 10C on 100-120 mesh Gas Chrom Q; column temp = 190 C; detector and injector temp = 240 C; nitrogen flow rate = 20 ml/min.

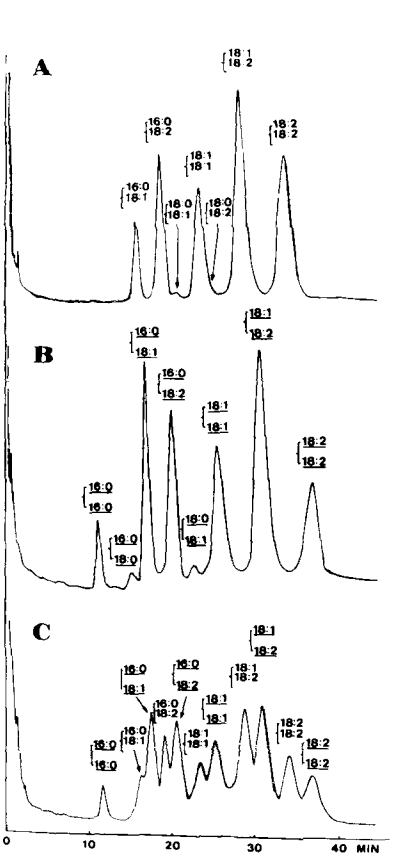

FIG. 2. Gas chromatographic resolution of corn oil diacylglycerols as TMS ethers on SILAR 10C. (A) 1,2(2,3)-diacylglycerols; (B) 1,3-diacylglycerols; (C) ca. 1:1 mixture of A and B. GLC conditions: column, glass tubes (2 m x 3 mm id) packed with 5% SILAR 10C on 100-120 mesh Gas Chrom Q; column temp = 270 C; detector and injector temp = 280 C; nitrogen flow rate = 13 ml/min.

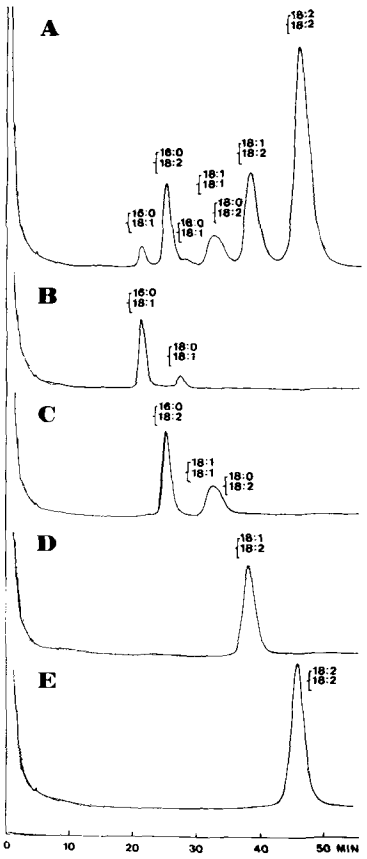

FIG. 3. Gas chromatographic resolution of safflower oil diacylglycerols as acetates on SILAR 10C. (A) Total 1,2(2,3)-diacylglycerols; (B) monoenes; (C) dienes; (D) trienes; (E) tetraenes. The fractions B, C and D were separated from A by $AgNO_3$-TLC. GLC conditions: column, glass tubes (1.5 m x 3 mm id) packed with 3% SILAR 10C on 100-120 mesh Gas Chrom Q; column temp = 270 C; injector and detector temp = 300 C; nitrogen flow rate = 30 ml/min.

On the other hand, there were close linear relationships between the RTs of these acylglycerol derivatives and their carbon numbers under temperature-program conditions of 200-270 C at 1 C/min. Similar observations have been obtained in the GLC of TGs on SILAR 10C (5) and nonpolar JXR (18). The nonlinearity and linearity phenomena described above suggest the interaction between the liquid phases and the acylglycerols is significantly influenced by column temp.

Table V gives the separation factors of fatty acids, MGs and DGs on SILAR 10C and SILAR 5CP. Compared to SILAR 5CP, SILAR 10C shows high separation factors for homologs differing by one double bond and low separation factors for homologs differing by 2 carbons. This demonstrates that the ECLs of the unsaturated species on SILAR 10C are significantly different from that on SILAR 5CP. The separation factors of the homologs differing by 2 carbons for MG acetates on SILAR 10C are considerably lower than those of the corresponding TMS ethers and methyl esters (Table V). This is responsible for the characteristic elution order of the anchovy oil MG acetates (Fig. 5).

Effect of Column Temperature on Retention Data

The differences between the separation factors of the acetates and TMS ethers of MGs on SILAR 10C (Table V) progressively decrease with increasing column temp. For example, the separation factor of the acetates of monopalmitoyl- and monostearoylglycerols was 1.45 at 220 C and 1.31 at 260 C, whereas that of the corresponding TMS ethers was 1.67 at 190 C and 1.56 at 210 C. On the other hand, the small

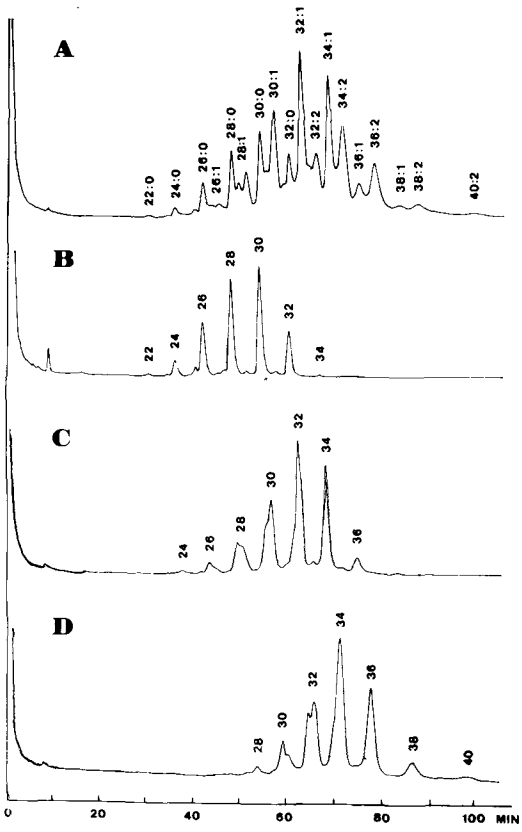

FIG. 4. Gas chromatographic resolution of sperm whale oil diacylglycerols as acetates on SILAR 10C. (A) Total 1,2(2,3)-diacylglycerols; (B) saturates; (C) monoenes; (D) dienes. The fractions B, C and D were separated from A by $AgNO_3$-TLC; column temp = 200-270 C at 1 C/min. Other GLC conditions as given in Figure 3.

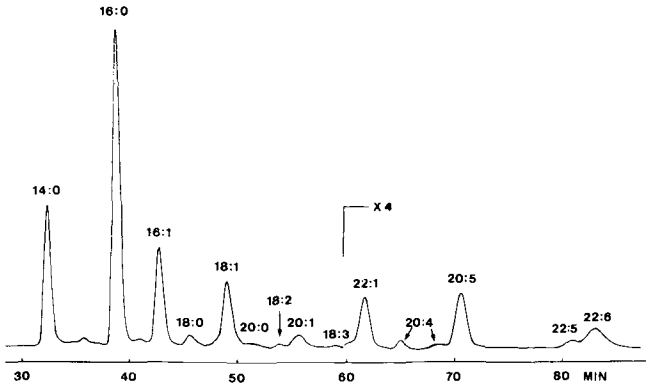

FIG. 5. Gas chromatographic elution pattern of Japanese anchovy oil monoacylglycerols as acetates on SILAR 10C. GLC conditions: column, glass tubes (2 m x 3 mm id) packed with 5% SILAR 10C on 100-120 mesh Gas Chrom Q; column temp = 200-270 C at 1 C/min; injector and detector temp = 270 C; nitrogen flow rate = 20 ml/min.

TABLE II

Relative Retention Data of Monoacylglycerols on SILAR 10C[a]

Molecular species	Acetate		TMS ether			
			1(3)-Acyl		2-Acyl	
	RRT	ECL	RRT	ECL	RRT	ECL
14:0	0.7216	14.00	0.6046	14.00	0.5318	13.50
16:0	1.000	16.00	1.000	16.00	0.8814	15.50
18:0	1.388	18.00	1.672	18.00	1.474	17.51
20:0	1.911	20.00	2.785	20.00	2.467	19.53
22:0	2.630	22.00	4.676	22.00	4.147	21.54
24:0	3.630	24.00	7.971	24.00	---	---
16:1	1.222	17.22	1.174	16.62	1.019	16.07
18:1	1.661	19.12	1.875	18.47	1.649	17.95
20:1	2.267	21.07	3.097	20.41	---	---
22:1	3.067	22.95	5.063	20.30	---	---
18:2(n-6)	2.090	20.56	2.307	19.26	2.009	18.72
18:3(n-3)	2.679	22.11	2.946	20.22	2.564	19.68
20:4(n-6)	3.622	23.98	---	---	---	---
20:4(n-3)	4.288	25.03	---	---	---	---
20:5(n-3)	4.600	25.47	5.539	22.63	4.743	22.03
22:6(n-3)	7.022	28.09	9.814	24.79	8.322	24.16

[a]Monoacylglycerols prepared from linseed and Japanese anchovy oils were used to obtain these data. RRT = retention time relative to 1(3)-monopalmitoylglycerol; ECL = equivalent chain length; GLC conditions: column, glass tubes (2 m x 3 mm id) packed with 5% SILAR 10C on 100-120 mesh Gas Chrom Q; column temp = 240 C for acetates and 190 C for TMS ethers; nitrogen flow rates = 20 ml/min.

differences between the separation factors of the TMS ethers and acetates of DGs on SILAR 10C are mainly caused by structural differences of the derivatives. This effect is more apparent for the separation factors of methyl esters and MG-TMS ethers determined under the same GLC conditions. In the GLC of MGs on SILAR 5CP (11), the elution orders of the TMS ethers and acetates are the same at different column temp, in contrast to those on SILAR 10C. This difference in the elution orders on SILAR 10C and 5CP can be explained considering the effect of column temp on the ECLs for SILAR 5CP is significantly less than for SILAR 10C (see Table VI).

In previous papers (2,4,5), it was reported that the ECLs of wax and steryl esters on SILAR 10C were more dependent upon column temp than ECLs of fatty acid methyl esters and TGs. The effect has been represented by Δ_tECL, an increase of ECL for one degree raising of column temp. Table VI compares Δ_tECL of fatty acid methyl esters on several polar cyanosiloxanes, and Table VII compares Δ_tECL of various derivatives of fatty acids on SILAR 10C. As expected, the Δ_tECL for MG and DG derivatives was nearly the same as for TGs and methyl esters, and was considerably lower than for wax and steryl esters. These results can be explained by the higher polarity of the acylglycerol derivatives, TGs and methyl esters than that of wax and steryl esters. The Δ_tECL for MG acetates was slightly higher than for the corresponding TMS ethers. This mainly results from the difference in the column temp used. The Δ_tECL generally increases with degree of unsaturation of methyl esters and polarity of liquid phases (Table VI). These observations indicate the effect of column temp on the ECLs of unsaturated methyl esters and acylglycerols is remarkable on the highly polar liquid phases SILAR 10C and OV-275. The effect of column temp on the resolution of methyl esters on OV-275 has been discussed elsewhere (19). In the GLC of DG-TMS ethers on 5% SILAR 10C, one double bond approximately corresponds to one acyl carbon in ECL. Therefore, the diene peaks almost overlap with the peaks resulting from saturates with 2 more carbons. A similar observation has been obtained in GLC of wax esters on SILAR 10C (2). In the GLC, hexadecyl linoleate (34:2) and octadecyl stearate (36:0) showed a single overlapped peak at 230 C but were resolved almost completely at 210 C. In this study, DGs (as acetates) with nearly the same ECL values

TABLE III

Relative Retention Data of Diacylglycerols on SILAR 10C[a]

Molecular species			Acetate		TMS ether			
					1,3-Diacyl		1,2(2,3)-Diacyl	
			RRT	ECL	RRT	ECL	RRT	ECL
28:0	14:0	14:0	0.5895	28.00	0.5754	28.00	0.5422	27.5
30:0	---	---	0.7662	30.00	0.7573	30.00	0.7129	29.56
32:0	16:0	16:0	1.000	32.00	1.000	32.00	0.9440	31.59
34:0	---	---	1.307	34.00	1.332	34.00	1.258	33.60
36:0	18:0	18:0	1.724	36.00	1.788	36.00	1.691	35.62
38:0	---	---	2.287	38.00	2.399	38.00	2.286	37.67
40:0	---	---	3.069	40.00	3.240	40.00	3.104	39.71
42:0	---	---	4.177	42.00	4.436	42.00	4.276	41.77
34:1	16:0	18:1	1.525	35.11	1.504	34.82	1.415	34.41
36:1	18:0	18:1	2.017	37.11	2.021	36.83	1.892	36.38
34:2	16:0	18:2	1.815	36.37	1.777	35.95	1.659	35.49
36:2	18:1	18:1	2.292	38.01	2.264	37.61	2.127	37.18
36:2	18:0	18:2	2.355	38.20	2.333	37.81	2.164	37.30
36:3	18:1	18:2	2.730	39.21	2.676	38.73	2.492	38.25
36:4	18:2	18:2	3.281	40.43	3.184	39.89	2.950	39.38
36:5	18:2	18:3	3.999	41.72	3.828	41.06	3.543	40.57
36:6	18:3	18:3	4.903	43.1	4.629	42.3	4.296	41.80

[a]Diacylglycerols prepared from safflower, corn, linseed and hydrogenated Japanese anchovy oils were used to obtain these data. RRT = retention time relative to 1,3-dipalmitoylglycerol; ECL = equivalent chain length; GLC conditions: column, glass tubes (1.5 m x 3 mm id) packed with 3% SILAR 10C on 100-120 mesh Gas Chrom Q for acetates and glass tubes (2 m x 3 mm id) packed with 5% SILAR 10C on 100-120 mesh Gas Chrom Q for TMS ethers; column temp = 270 C; nitrogen flow rate = 20 ml/min for acetates and 13 ml/min for TMS ethers.

TABLE IV

Comparison of Chain Lengths for Saturated Diacylglycerols Calculated by Linear and Quadratic Methods[a]

Molecular species	Acetate		TMS ether	
	Linear[b]	Quadratic[c]	Linear	Quadratic
28:0	28.25	27.84	28.30	27.98
30:0	30.14	29.94	30.17	30.01
31:0	31.13	31.00	31.05	30.96
32:0	32.06	32.00	32.05	32.00
33:0	33.07	33.03	32.99	32.97
34:0	34.00	34.00	34.00	34.00
35:0	35.09	35.09	35.00	35.00
36:0	36.00	36.00	36.00	36.00
38:0	38.04	37.98	38.00	37.94
40:0	40.17	39.98	40.04	39.88
42:0	42.40	42.01	42.17	41.86

[a]Calculated according to the procedure described by Nelson (17). GLC conditions as given in Table III.

[b]Relative retention times of 34:0 and 36:0 were used as reference points.

[c]Relative retention times of 32:0, 34:0 and 36:0 were used as reference points.

TABLE V

Separation Factors of Fatty Acids, Monoacylglycerols and Diacylglycerols on SILAR 10C and SILAR 5CP[a]

	Fatty acid		Monoacylglycerol				Diacylglycerol		
	Methyl ester		Acetate		TMS ether		Acetate	TMS ether	
Molecular species	10C (190 C)	5CP (185 C)	10C (240 C)	5CP (248 C)	10C (190 C)	5CP (220 C)	10C (270 C)	10C (270 C)	5CP (270 C)
Monoene/Saturate[b]	1.20	1.12	1.20	1.10	1.12	1.10	1.17	1.13	1.10
Diene/Monoene	1.29	1.18	1.26	1.14	1.23	1.16	1.14	1.12	1.10
Triene/Diene	1.33	1.26	1.28	1.17	1.28	1.21	1.19	1.18	1.16
Tetraene/Triene	---	---	---	---	---	---	1.20	1.19	1.15
Pentaene/Tetraene	---	---	---	---	---	---	1.22	1.20	1.17
Hexaene/Pentaene	---	---	---	---	---	---	1.23	1.21	1.18
C_{n+2}/C_n[c]	1.61	2.08	1.39	1.60	1.67	1.79	1.32	1.34	1.52

[a]Fatty acids (16:0, 18:0, 18:1, 18:2, 18:3), 1(3)-monoacylglycerols (16:0, 18:0, 18:1, 18:2, 18:3) and 1,3-diacylglycerols (16:0-18:0, 18:0-18:0, 18:0-18:1, 18:1-18:1, 18:1-18:2, 18:2-18:2, 18:2-18:3, 18:3-18:3) were used to obtain these data. Values on SILAR 10C were calculated from the RRT in Tables II and III; those on SILAR 5CP were calculated from the Myher and Kuksis data (9,11).

[b]Separation factors of species with acyl group of same carbon number.

[c]Separation factors of saturated species. n = Carbon number of acyl group.

TABLE VI

Comparison of Δ_tECL of Fatty Acid Methyl Esters on Cyanosiloxane Liquid Phases[a]

Fatty acid	SILAR 5CP 190-230[c]	SILAR 7CP 170-210	SILAR 10C[b] 150-190	OV-275 150-190
18:1	0.002	0.003	0.007	0.007
18:2	0.003	0.005	0.011	0.012
18:3	0.004	0.007	0.013	0.019

[a]Δ_tECL: increase of ECL per one degree rise in column temp. GLC columns and carrier gas flow rate as given in Table I.

[b]The data were selected from our previous paper (2).

[c]190-230 Indicates the Δ_tECL was calculated from ECLs at 190 and 230 C.

TABLE VII

Comparison of Δ_tECL of Various Derivatives of Fatty Acids on SILAR 10C[a]

Molecular species	Monoacylglycerol[b]		Diacylglycerol[c]		Triacylglycerol[d]	Cholesteryl ester[e]	Wax ester[f]
	Acetate 240-260[g]	TMS ether 190-210	Acetate 250-270	TMS ether 250-270	250-270	250-270	210-230
Monoene	0.007	0.007	0.007	0.010	0.003	0.016	---
Diene	0.015	0.010	0.009	0.012	0.011	0.023	0.028
Triene	0.022	0.014	0.026	0.025	0.020	0.032	0.037
Tetraene	---	---	0.034	0.032	0.024	---	---

[a]Δ_tECL: increase of ECL per one degree rise in column temp. The data d, e, and f were selected from our previous papers (2,4,5). GLC conditions for b and c are given in Tables II and III.

[b]1(3)-monoacylglycerols (18:1, 18:2, 18:3).

[c]1,3-diacylglycerols (16:0-18:1, 18:1-18:1, 18:1-18:2).

[d]C48 triacylglycerols from coconut oil.

[e]Oleate, linoleate and linolenate.

[f]Hexadecyl linoleate and octadecyl linolenate.

[g]240-260 Indicates the Δ_tECL was calculated from ECLs at 240 and 260 C.

at 270 C, such as 36:2 (dioleoylglycerol) and 38:0, and 34:2 and 36:0 (as TMS ethers) could not in practice be resolved at 250 C. These phenomena can be explained by the significantly lower Δ_tECL for DG derivatives than for wax esters (Table VII).

In this study, we found the ECLs of DG acetates on SILAR 10C can be calculated by the following equation:

$$ECL_{DG\ acetate} = ECL_{MG\ acetate} + ECL_{methyl\ ester},$$

where the degree of acyl group unsaturation of the MG acetate must be higher than or equal to that for the methyl ester. The ECLs of the acetates of monooleoyl-, monolinoleoyl- and monolinolenoylglycerols on the 3% SILAR 10C column (240 C) were 19.06, 20.46 and 21.93, respectively, whereas those of the methyl esters of oleic, linoleic and linolenic acids on the 5% column (190 C) were 18.79, 19.91 and 21.07, respectively. Using these values, for example, the ECL of 1(3)-linoleoyl-2-linolenoylglycerol acetate (18:2-18:3) can be calculated as follows: $ELC_{18:2\text{-}18:3} = 21.93 + 19.91 = 41.84$. The results of these calculations are shown in Table VIII. The differences between the calculated and found values mainly result from the difference of the column temp effect on the ECLs of MG acetates and methyl esters. To obtain fair agreement between these values, the optimum column temp for MG acetates and methyl esters were 240 and 190 C, respectively, when DG acetates were analyzed at 270 C.

Interrelationships of Retention Data of Positional Isomers

In the GLC of MG-TMS ethers on SILAR 5CP (11), the separation factors of the posi-

TABLE VIII

Comparison of Found and Calculated ECLs of Diacylglycerol Acetates

Molecular species			
1(3)-Position	2-Position	Found[a]	Calculated[b]
16:0	18:1	35.11	35.06
18:0	18:1	37.11	37.06
16:0	18:2	36.37	36.46
18:1	18:1	38.01	37.85
18:0	18:2	38.20	38.46
18:1	18:2	39.21	39.25
18:2	18:2	40.43	40.37
18:2	18:3	41.72	41.84
18:3	18:3	43.1	43.00

[a]Selected from the data in Table III.

[b]Calculated by summing ECLs of fatty acid methyl ester and monoacylglycerol acetate as explained in Results and Discussion.

tional isomers are the same for all species with 14-22 acyl carbons and 0-6 double bonds, regardless of the chain lengths and degree of unsaturation. On SILAR 10C, the separation factors of positional isomers progressively decrease with increasing carbon number (1.141→1.128 for saturated C14-C24 MGs and 1.065→1.037 for saturated C28-C42 DGs), and the separation factors of unsaturated isomers are higher than those of saturated isomers (1.149, 1.134, 1.081 and 1.056 for monolinolenoyl-, monostearoyl-, dilinolenoyl- and distearoylglycerols, respectively) (Tables II and III). In addition, the peak separation (20) for unsaturated isomers was considerably greater than for saturated isomers (24.9% for distearoylglycerols and 55.3% for dioleoylglycerols at 270 C in a mixture of 1:2 isomer ratio). These phenomena for the separation of positional isomers were more apparent for DGs than MGs.

Although the separation factors of saturated isomers on SILAR 10C are not constant for all species as described above, the ECLs of unsaturated isomers used in this study can be correlated by the following equations:

$$ECL_{1(3)\text{-MG}} = ECL_{2\text{-MG}} + 0.56 \text{ and}$$

$$ECL_{1,3\text{-DG}} = ECL_{1,2(2,3)\text{-DG}} + 0.47,$$

where the constants 0.56 and 0.46 are average values obtained for all unsaturated acylglycerols listed in Tables II and III. The standard deviation was ± 0.04 and ± 0.03, respectively. The constants indicate the separation factors for DG isomers are slightly lower than for MG isomers (0.1 carbon). Using these equations, the ECLs of 1(1,3)-acylglycerols can be calculated from the ECLs of the corresponding 2(1,2)-acylglycerols within a precision of one decimal.

Resolution of Diacylglycerol Acetates and Triacylglycerols of the Same Molecular Weight

GLC resolution of TG isomers of the same MW but different fatty acids, such as trilauroylglycerol and 1,2-dimyristoyl-3-caproylglycerol has been examined previously (7,21). In this study, the resolution on SILAR 10C was investigated with the saturated DG acetates given in Table III and saturated C28-C42 TGs obtained from coconut oil (5). Under tempera-

TABLE IX

Compositions of Safflower Oil Diacylglycerols As Acetates

Molecular species		Peak area %[a]	
1(3)-Position	2-Position	SILAR 10C[b]	OV-17[c]
16:0	18:1	1.73 ± 0.33	
16:0	18:2	12.10 ± 0.33	
	Total C34	13.83	13.85 ± 0.11
18:0	18:1	0.14 ± 0.04	
18:1	18:1	7.85 ± 0.40	
18:0	18:2		
18:1	18:2	20.97 ± 0.11	
18:2	18:2	57.21 ± 0.99	
	Total C36	86.17	86.15 ± 0.11

[a]Mean ± standard deviation of five analyses.

[b]GLC conditions as given in Figure 4.

[c]GLC conditions: column, glass tubes (0.5 m x 3 mm id) packed with 2% OV-17 on 80-100 mesh Gas Chrom Q; injector, detector and column temp = 300 C; nitrogen flow rate = 80 ml/min.

ture-program of 200 to 270 C at 1 C/min, parallel lines were obtained in the plot of RT against total number of carbon atoms. This parallel relationship showed that TG of total carbon number n has RT corresponding to that of DG acetate of total carbon number n - 1.6. From these results, one may expect the resolution of the isomers of natural TGs having the same MW but different fatty acids. However, the resolution has not been observed on SILAR 10C or any other column.

Quantitative Analysis of Diacylglycerol Acetates

Table IX compares the compositions of safflower oil DGs (as acetates) obtained by GLC on SILAR 10C and OV-17. The OV-17 column gave only 2 peaks of C34 and C36. The good agreement of peak area percentages on the polar and nonpolar columns indicates the reliability of the GLC on SILAR 10C for quantitative analysis of DG acetates.

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Effects of Three Dietary Fats on Plasma Lipids and Lipoproteins in Fasting and Post-prandial Humans after a Short-term Diet

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ABSTRACT

The effects of 3 dietary fats (olive oil, canbra oil and butter) on the fatty acids of blood lipids and on serum lipoproteins were compared in 6 healthy adult outpatients, after a 6-day normocaloric diet including 35% of the studied fat. Important, although incomplete, changes appeared in the fatty acid composition of the various serum lipids and in the composition and distribution of serum lipoproteins. These changes probably result from the degree of saturation of the fat ingested. Moreover, differences were observed among individual subjects. Genetic differences, which are important in clinical practice, are stressed in connection with risks of vascular diseases and hyperlipidemia and affect intestinal fat absorption and lipoprotein metabolism.

INTRODUCTION

The degree of saturation of dietary fats is known to play a very important part in lipidemia, and in man, long-term ingestion of polyunsaturated fats has been shown to have multiple effects. These include a reduction of cholesterolemia and triglyceridemia (1-6), changes in plasma triglyceride acyl groups (7) and an increased lipoprotein fluidity (8). Plasma cholesterol levels have been observed to decrease in both normal and hyperlipidemic subjects, but little is known about the mechanisms which reduce lipidemia. There are fairly large differences in the responses observed by different authors, but the lipidemia-reducing mechanisms most often suggested are the following: (a) increased cholesterol and bile acid excretion (9-11). Some authors have expressed doubt about this possibility, Thus, Grundy and Ahrens found no increase in fecal sterols in subjects on a polyunsaturated fat diet (12); (b) a diminution of the low density lipoproteins (LDL) accompanied by a change in lipid and apoprotein distribution in these LDLs (5); (c) reduced triglyceridemia in hypertriglyceridemic patients accompanied by decreased serum cholesterol, resulting from a polyunsaturated fat diet (4). This prompted Grundy's hypothesis that such a diet induced a change in the metabolism of very low density lipoproteins (VLDL) in these subjects. It is probable however, that several mechanisms combine to reduce cholesterolemia after ingestion of a polyunsaturated fat diet.

Recent work with the rat (13) has shown that changes in the fatty acid composition of plasma lipids occurred during the first days of the diet. In addition, the results of lipid overloading tests in man showed that they were followed, a few hours later, by changes in platelet aggregation and in lipid platelet composition (14,15).

This led us to compare the effects of 3 very different dietary fats: butter, olive oil and canbra oil (rapeseed oil with a low erucic acid content) on lipidemia, serum fatty acid composition and serum lipoprotein composition and distribution, after ingestion of these fats by normolipemic subjects for six days.

MATERIALS AND METHODS

Subjects

We tested 6 healthy adult outpatients (5 women and 1 man) whose average age was 27.3 (ages ranged from 26 to 36). Their average weight was 58.16 kg and their height ranged from 1.60 to 1.62 m (Table I). Fasting serum lipid levels were normal in these subjects (Table III).

Diets

The test procedure for each fat was as follows (Fig. 1): For 6 days, each subject was given a standard diet with a daily intake of 1,500 to 1,800 calories, depending on their feeding habits. Subjects' weights remained stable throughout the investigation.

The fat tested was the only visible fatty component of the diet, and the amount of fat ingested comprised 35% of total calorie intake, the remainder consisting of 40% glucide and 25% protein calories.

On the morning of the 7th day, a first blood sample was taken from fasting subjects; a second was taken 3 hr after subjects had ingested a meal comprising 700 calories, including 20 g of the fat administered during the preceding 6 days.

In addition to the above procedure just described, a control sample was taken from fasting subjects while they were not following

TABLE I

Characteristics of the Tested Subjects

	Sex	Age (years)	Height (meter)	Weight (kilos)	Diet (calories)
GIR...	F	26	1,60	56	1,500
NAV...	F	36	1,60	47	1,500
WIN...	F	25	1,62	53	1,500
DET...	F	25	1,62	60	1,500
ADU...	F	27	1,62	63	1,800
ABS...	M	25	1,60	70	1,800

any of the specified diets. During this control period, their daily calorie intake was the same as that in the diets (1,500 to 1,800 calories), and comprised the following: 40-45% glucide calories, 20% protein calories and 35-40% lipid calories. The lipids were a mixture of butter, arachide oil and saturated fats. The saturated fats made up about 60% of the fats in this mixture; the remaining 40% were unsaturated fats (20% monounsaturated and 20% polyunsaturated. There was a 2-month interval between each test. The fatty acid composition of each fat studied was known (Table II).

Plasma Lipoproteins

Plasma lipoproteins were separated by ultracentrifugation according to Havel et al. (16) into VLDL (d: 1.006), intermediate LDL (ILDL) (d: 1.006-1.019), LDL (d: 1.019-1.063), and high density lipoproteins (HDL) (d: 1.063-1.21). Each category of lipoproteins was centrifuged a second time at its density and dialyzed for 48 hr against a physiological saline solution (pH 7.4) containing tris (hydroxymethyl) aminomethane, (Tris) and 0.01% ethylenediaminetetraacetate (EDTA). The purity of the lipoproteins thus obtained was confirmed by immunoelectrophotoresis and agarose gel electrophoresis. In each lipoprotein class, cholesterol was measured by gas chromatography (17); phospholipids were determined according to Van Gent and Roseleur (18), triglycerides using the Biolyon Laboratories Triglyceride B test Wako kit, and proteins according to Lowry et al. (19).

Fatty Acid Levels

In all serum lipids (triglycerides, esterified cholesterol, phospholipids and free fatty acids), fatty acids were measured as follows: lipids were extracted from 1 ml serum by Folch's technique (20), using 15 ml chloroform/methanol (2:1). The extract was evaporated to dryness under nitrogen and resuspended in a small amount of chloroform. Lipids were then separated by thin layer silica gel chromatography in 2 successive solvent systems: a diethylether/acetic acid/petroleum ether system (100:3:97), and a diethylether/petroleum ether system (6:194). The various lipids were identified using control serum lipids that were visualized by iodine vapor. A known amount of heptadecanoic acid derivative, used as the internal standard in gas chromatography (GC), was deposited on each lipid fraction which was then removed by scraping.

The fatty acids in the different lipids were hydrolyzed and methylated with a methanol solution containing 2% sulfuric acid and 0.2% benzene (21).

GC was applied isothermally to methylated fatty acids on a Girdel 3000 chromatograph equipped with a flame ionization detector. The 2 m stainless steel column used was packed with butanediol-succinate (BDS) on WAW 80-100 chromosorb. Nitrogen was the carrier gas. Temperatures were 180 C for the column, 220 C for the injector and 230 C for the detector.

Methylated fatty acids were identified by comparing their retention times with those of control fatty acids. Peak areas were determined manually. Since the different acid response coefficients were assumed to be equal, the amount of each fatty acid was determined in reference to the internal standard.

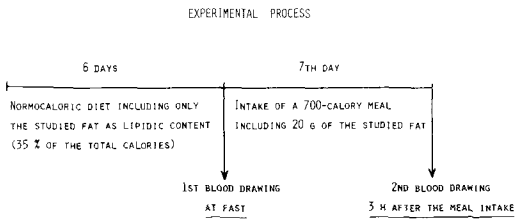

FIG. 1. Diagram for the study of fat's effect on human serum lipids.

Statistical Analysis

(a) The Student-Fischer test was used to compare the means of paired values in the experimental and control series; (b) a discriminant analysis test was applied to measurements of several parameters in a group of individuals divided into previously defined subgroups (in this case, according to the type of fat ingested) and to the measurements of the different fatty acid levels. This analysis was designed to show whether differentiation among the groups of individuals studied can be based on determination of these variables (22).

RESULTS

Serum Lipid Fatty Acids

Overall results. After 6 days on the canbra and olive oil diets, fasting subjects showed decreased fatty acids for all serum lipid fractions (Table III). On the other hand, the butter diet caused increased fatty acid levels. This increase was significant for cholesteryl esters and phospholipids when butter diet results were compared to results for the olive or canbra oil diets; the increase was not significant when butter diet samples were compared to control samples.

TABLE II

Fatty Acid Composition of the Studied Fats (%)

		Butter	Olive oil	Canbra oil
Fatty acids with less than 16 carbon atoms		25.5 (14:0 = 9.5)	0	0
Saturated fatty acids	16:0	24.3	8.0	4.4
	17:0	1.8	0	0
	18:0	14.7	3.0	1.3
Monounsaturated fatty acids	16:1	3.2	0.7	0.2
	17:1	0.4	0	0
	18:1	27.6	74.2	63.7
Polyunsaturated fatty acids	18:2	1.3	13.5	21.4
	18:3	1.0	0.6	7.0
Fatty acids containing 20-24 carbon atoms		0.2	0	2

TABLE III

Fatty Acid Content of the Different Serum Lipids
(Mean values in μg/ml of serum ± the standard deviation)

Diets		Triglycerides	Free fatty acids	Cholesteryl esters	Phospholipids
Control		217 ± 34	108 ± 16	496 ± 56	395 ± 46
Butter	Fasting	294 ± 44 (a)	136 ± 22	552 ± 43 (c) (d)	494 ± 24 (e) (f)
	After test meal	362 ± 72	102 ± 26	422 ± 26	408 ± 20
Olive oil	Fasting	210 ± 23	119 ± 10 (b)	377 ± 58 (d′)	361 ± 41 (f′)
	After test meal	303 ± 40	82 ± 15	428 ± 26	358 ± 17
Canbra oil	Fasting	185 ± 26 (a′)	83 ± 9 (b′)	324 ± 49 (c′)	303 ± 27 (e′)
	After test meal	464 ± 115	98 ± 13	478 ± 43	421 ± 50

The significative differences (Student t-test) are the following:
(a), (a′): $p \leq 0.05$
(b), (b′): $p \leq 0.05$
(c), (c′): $p \leq 0.01$
(d), (d′): $p \leq 0.01$
(e), (e′): $p \leq 0.001$
(f), (f′): $p \leq 0.05$

In samples taken postprandially, the fatty acid level in the triglyceride fraction increased, regardless of the diet. In the 3 other lipid fractions (cholesteryl esters, phospholipids and free fatty acids), the fatty acid content decreased after the butter diet and increased after the canbra and olive oil diets.

Fatty acid composition. Fatty acid measurements in the 4 lipid fractions led to the following observations: (a) lauric acid (12:0) was only present in triglycerides and free fatty acids in very small amounts; (b) linolenic acid (18:3) was mostly evident in canbra diet subjects; and (c) arachidonic acid (20:4) was present only in cholesteryl esters and phospholipids.

Assays of samples taken from fasting subjects after a 6-day diet showed that each serum lipid fraction kept its characteristic fatty acid composition regardless of the fat ingested during the preceding days.

The decreased serum lipids observed after the olive and canbra oil diets affected fatty acids as a whole, and the composition of the different lipid fractions was very close to that of the control sample. However, the increase in serum lipids after the butter diet was essentially caused by the increased intake of saturated fatty acids and oleic acid (18:1). This increase was greatest for triglycerides and was clearly shown by discriminant analysis (Fig. 2a).

The following results were observed after absorption of the fatty diet. Triglycerides: after butter ingestion, there was a rise in the level of saturated fatty acids (myristic 14:0; palmitic 16:0; and stearic 18:0), as well as a notable increase in oleic acid (18:1). This was especially clear after discriminant analysis (Fig. 2b). After canbra oil ingestion, an increase was observed in unsaturated fatty acids, i.e., palmitoleic acid (16:1); oleic acid (18:1); and linoleic acid (18:2) (Fig. 2b). Ingestion of the olive oil diet caused increased oleic acid levels (18:1) (Fig. 2b).

Examination of triglyceride levels in each subject showed their responses varied considerably. Thus, 3 out of 6 reacted strongly to the fatty diet, but in one of the remaining 3, there was only a light change in fatty acid composition, regardless of the fat ingested. In the discriminant analysis diagram (Fig. 2a and 2b), all points applying to this subject are grouped in the lower part of the area concerned. The responses of the 2 remaining subjects were of medium intensity.

In the other serum lipid fractions (cholesteryl esters, phospholipids and free fatty acids), the butter diet caused a reduction in all fatty acids. The slight increase in these acids

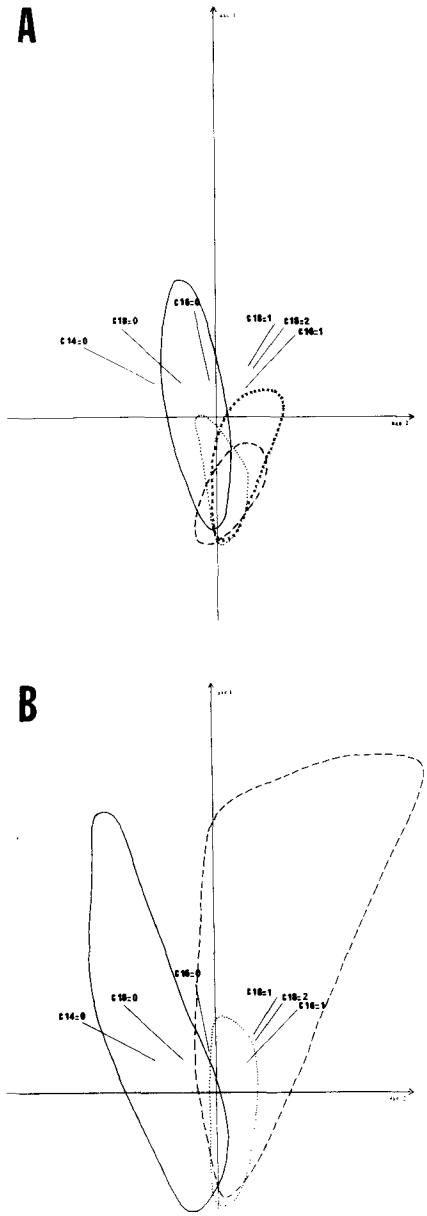

FIG. 2. Discriminant analysis between the control and the 3 tested diets for serum fatty acids of 6 subjects: (a) after a 6-day diet containing one of the 3 tested fats; (b) 3 hr after the intake of a meal containing the same fat. xxxxxx: control with normal diet; ———: butter diet; .....: olive oil diet; ------: canbra oil diet. Axes 1 and 2 are those which have the highest discriminant power, i.e., those which best determine the groups of data as a function of the diets (22).

after the olive and canbra oil diets chiefly affected unsaturated fatty acids. Nevertheless, free fatty acids diminished after the olive oil diet but rose slightly after the canbra oil diet.

Serum Lipoproteins

Comparison of postdiet lipoprotein composition with that of control samples produced the following results (Tables IV, V, VI and VII).

Fasting subjects. The butter diet caused a large increase in the different β-lipoprotein levels: + 32% for VLDL, + 30% for ILDL and + 15% for LDL. On the other hand, the HDL level dropped by 14%. In addition, β-lipoprotein composition changed: cholesterol rose perceptibly but not significantly in VLDL and ILDL, and slightly in LDL. Triglycerides diminished perceptibly although not significantly in VLDL and ILDL. Phospholipids rose significantly in VLDL. The olive oil diet raised the level of VLDL (+ 13%), LDL (+ 25%) and HDL (+ 21%) but reduced the ILDL level (- 27%). Lipoprotein composition was virtually unchanged by this diet. The canbra oil diet increased VLDL (+ 20%) and HDL (+ 5%), and slightly reduced ILDL. The LDL level remained constant and variations in lipoprotein composition were very small.

Postprandial subjects. Regardless of the fat ingested, VLDL increased 30-40%. Butter considerably reduced LDL (- 40%) but lipoprotein composition was practically unaltered by absorption of a fatty meal.

DISCUSSION

Our results show that a 6-day diet can cause obvious changes in the level and composition of blood lipids and in the distribution of the different lipoprotein classes. These changes probably result from the degree of saturation of the fat ingested.

After 6 days of a normocaloric diet including saturated fats only (butter), a large increase in all fatty acid levels was observed, mainly because of the increased saturated fatty acids in serum lipids. Meals which included olive or canbra oil as the only visible fat were followed by a reduction in blood fatty acids as a whole. The decrease was greater after absorption of canbra oil, which contains a higher percentage of polyunsaturated acids.

Various mechanisms have been suggested to explain why diets rich in polyunsaturated fatty acids reduce serum lipids, particularly cholesterol. Such diets certainly increase bile excretion of sterols, but this is just one of several explanations. Spritz and Mishkel (5) pointed out that saturated and unsaturated fatty acids have very different areas in the serum lipoprotein molecule. The more double bonds a fatty acid contains, the larger the area occupied. This causes a change in the spacial configuration of lipids incorporating fatty acids. According to Spritz and Mishkel, the presence of polyunsaturated fatty acids determines the reduced number of lipid molecules associated with lipoproteins. Our results strengthen this hypothesis, since the blood lipid level increased after a butter diet and decreased after a canbra or olive oil diet; the reduction was greater for canbra oil, which is richer in polyunsaturated fatty acids.

Samples taken during the postprandial period showed that serum triglycerides were increased greatly in 5 subjects and slightly modified in 1 for all 3 dietary fats. This increase, perceptible 3 hr after the meal, resulted from the presence of chylomicrons and VLDL produced by intestinal cells. Levels for other esterified fatty acids (cholesteryl esters and phospholipids) increased 3 hr after the olive and canbra oil diets, but tended to decrease after the butter diet.

Cholesteryl esters and phospholipids are transported mainly by the VLDL resulting from VLDL metabolization (they are also transported by HDL, but the HDL's slow turnover means they certainly are not involved in the postprandial period).

These differences suggest variations in VLDL catabolism, since this process is perhaps slower after a meal containing butter; the reduction in cholesteryl esters and phospholipids could result from delayed LDL formation.

Possible explanations for the decreased serum lipids are: (a) a defect in VLDL hydrolysis by serum lipases, or (b) a metabolic blocking in liver or adipose tissue. A study to investigate this issue is in progress in our laboratory.

It should be stressed that fairly clear differences were observed among individual subjects regarding the level and composition of serum fatty acids after ingestion of a meal, regardless of the fat absorbed. This probably explains certain divergences reported in the literature concerning the effects of polyunsaturated fats on cholesterolemia and lipidemia. Genetic differences, important in clinical practice are connected to the risk of vascular disease and hyperlipidemia and affect intestinal fat absorption (particularly lipoprotein metabolism).

The composition of the different lipoprotein categories was not greatly altered by a 6-day normocaloric diet containing olive or canbra oil

TABLE IV

VLDL Composition

Diet		Apoprotein in mg/ml	VLDL composition (%)			
			Triglycerides	Cholesterol	Phospholipids	Proteins
Control		0.027 ± 0.006	52.2 ± 10.2 (a)	14.6 ± 3.8 (b)	14.8 ± 8.2	18.3 ± 4.3
Butter	Fasting	0.040 ± 0.006	27.4 ± 8.8 (a′)	30.5 ± 4.6 (b′)	21.3 ± 4.7	20.8 ± 4.7
	After test meal	0.057 ± 0.012	25.1 ± 10.7	22.6 ± 7.3	33.5 ± 13.1	18.8 ± 4.3
Olive oil	Fasting	0.032 ± 0.009	45.2 ± 6.2	17.2 ± 2.2	17.3 ± 5.1	20.2 ± 3.1
	After test meal	0.054 ± 0.013	48.0 ± 8.3	16.8 ± 2.8	14.4 ± 3.9	20.8 ± 4.0
Canbra oil	Fasting	0.035 ± 0.007	55.9 ± 4.6	17.2 ± 2.2	10.5 ± 2.9	16.5 ± 1.4
	After test meal	0.057 ± 0.008	48.6 ± 3.3	19.4 ± 1.9	14.4 ± 1.7	17.6 ± 2.1

The significant differences (Student t-test) are the following: (a), (a′): $p < 0.05$; (b), (b′): $p < 0.05$

TABLE V

ILDL Composition

Diet		Apoprotein in mg/ml	ILDL composition (%)			
			Triglycerides	Cholesterol	Phospholipids	Proteins
Control		0.015 ± 0.003	46.3 ± 3.305	11.5 ± 2.57	16.5 ± 2.24	25.7 ± 4.00
Butter	Fasting	0.021 ± 0.003	38.2 ± 5.75	15.2 ± 8.73	19.6 ± 4.69	27.1 ± 4.24
	After test meal	0.022 ± 0.007	39.3 ± 9.5	15.2 ± 4.81	24.9 ± 20.3	20.6 ± 4.32
Olive oil	Fasting	0.012 ± 0.003	49.0 ± 10.03	12.4 ± 4.20	17.1 ± 4.49	25.1 ± 3.96
	After test meal	0.022 ± 0.007	38.3 ± 10.28	9.6 ± 4.61	22.0 ± 7.06	30.1 ± 4.04
Canbra oil	Fasting	0.015 ± 0.004	45.2 ± 4.90	11.1 ± 7.10	10.4 ± 2.12	33.4 ± 3.26
	After test meal	0.018 ± 0.003	39.4 ± 5.14	12.8 ± 6.16	12.1 ± 3.02	35.7 ± 7.87

TABLE VI

LDL Composition

Diet		Apoprotein in mg/ml	LDL composition (%)			
			Triglycerides	Cholesterol	Phospholipids	Proteins
Control		0.415 ± 0.070	9.1 ± 1.2	42.2 ± 3.1	16.8 ± 2.1	31.6 ± 2.9
Butter	Fasting	0.490 ± 0.051	8.2 ± 1.2	50.1 ± 5.4	11.8 ± 1.7	29.8 ± 3.8
	After test meal	0.330 ± 0.085	10.4 ± 2.1	53.0 ± 6.7	14.2 ± 3.5	22.3 ± 5.9
Olive oil	Fasting	0.550 ± 0.074	7.7 ± 1.79	41.0 ± 3.3	20.9 ± 3.6	30.3 ± 2.2
	After test meal	0.549 ± 0.079	8.5 ± 1.8	42.5 ± 3.0	17.4 ± 2.8	31.6 ± 1.5
Canbra oil	Fasting	0.389 ± 0.078	7.6 ± 0.8	51.9 ± 3.7	16.5 ± 1.1	24.0 ± 4.2
	After test meal	0.428 ± 0.062	5.7 ± 0.5	44.3 ± 2.1	21.0 ± 1.7	29.0 ± 1.9

TABLE VII

HDL Composition

Diet		Apoprotein in mg/ml	HDL composition (%)			
			Triglycerides	Cholesterol	Phospholipids	Proteins
Control		0.912 ± 0.105	6.2 ± 1.3	25.2 ± 1.2	16.2 ± 2.5	52.4 ± 2.4
Butter	Fasting	0.798 ± 0.089	6.3 ± 0.8	23.3 ± 2.4	17.1 ± 1.5	53.4 ± 3.0
	After test meal	0.796 ± 0.075	7.4 ± 2.1	26.4 ± 4.6	16.2 ± 4.0	50.0 ± 2.7
Olive oil	Fasting	1.162 ± 0.223	6.9 ± 1.0	20.8 ± 3.2	15.3 ± 3.7	57.1 ± 4.2
	After test meal	1.009 ± 0.075	4.7 ± 1.3	21.7 ± 2.4	20.3 ± 4.0	53.3 ± 2.2
Canbra oil	Fasting	0.966 ± 0.088	5.5 ± 0.4	23.3 ± 1.2	16.6 ± 1.9	54.3 ± 1.3
	After test meal	0.894 ± 0.140	5.9 ± 0.8	23.9 ± 2.3	18.2 ± 1.9	52.0 ± 1.4

as the only visible fat. In contrast, the butter diet caused increased VLDL and ILDL cholesterol and decreased VLDL triglycerides. In this connection, saturated fats are known to be chiefly transported by small-sized molecules very similar to VLDL, with a high protein:triglyceride ratio, whereas unsaturated fats are transported by chylomicrons with a low protein:triglyceride ratio (23).

The most important changes observed after a 6-day diet concerned the distribution of the various lipoprotein classes. Thus, the butter diet caused a sharp increase in all β-lipoprotein levels and a reduction in α-lipoproteins. Unsaturated fat diets resulted in a moderate, parallel increase in β- and α-lipoproteins. The serum ratio HDL:VLDL+LDL was much lower for butter, a saturated fat, than for unsaturated fats, and was highest for canbra oil, a polyunsaturated fat. This might be important if, as certain studies have shown, the ratio is connected with the atherogenic power of lipidemia and varies in inverse proportion to this power.

Absorption of a fatty meal after 6 days of diet did not greatly alter the distribution of cholesterol, triglycerides, phospholipids and proteins in lipoproteins. The most notable change was the decreased VLDL cholesterol after the buffer diet.

In the distribution of the different lipoprotein classes, a rise in VLDL levels was noted 3 hr after the meal for all fats ingested. Butter ingestion caused a marked reduction in LDLs, which are produced by VLDL degradation.

In conclusion, administration to normolipemic subjects of a 6-day normocaloric diet containing one visible fat causes important, although not incomplete, changes in the fatty acid composition of the various serum lipids, and in the composition and distribution of serum lipoproteins. These changes are apparently linked to the degree of saturation of the fat ingested.

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Effect of Dietary Taurine on Bile Acid Metabolism in Guinea Pigs[1]

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ABSTRACT

The effect of oral administration of taurine (200-300 mg daily) on the metabolism of bile acids was studied in male guinea pigs which have predominantly glycine conjugated bile acids. The results were summarized as follows: (a) oral administration of taurine for 10 days increased taurine-conjugated bile acids and the ratio of glycine- to taurine-conjugated bile acids (G:T ratio) shifted from 3.95 to 0.19; (b) in taurine fed guinea pigs, the half-life of chenodeoxycholic acid (CDC) was about 40% shorter than that in controls and the fractional turnover rate increased by 70%; (c) the synthetic rate (mg/day/500 g body weight) of bile acids increased from 4.28 to 7.27 by taurine feeding; (d) hepatic cholesterol 7α-hydroxylase activity was increased 2.4-fold by taurine feeding; (e) the total pool size of bile acids did not change significantly but the amount of lithocholic acid in the caecum and large intestine increased by about 40%; (f) neither free cholesterol nor cholesterol ester levels in liver and serum changed significantly. Results of this study suggest that changing the G:T ratio in the bile acid conjugation pattern may influence the rate of hepatic bile acid synthesis.

INTRODUCTION

It was shown that taurine conjugates of different bile acids (cholic, deoxycholic and chenodeoxycholic acid [CDC]) have different effects on cholesterol 7α-hydroxylase, which is considered the rate-limiting enzyme in bile acid biosynthesis (1). These experiments suggest changes in the composition of bile acid pool produce changes in the rate of bile acid biosynthesis. However, the influence of changes in the bile acid conjugation pattern have not been investigated.

Bile acids in guinea pigs are conjugated mostly with glycine. The hepatic taurine concentration in human liver is a major determinant of the proportion of bile acids conjugated with taurine (2) and taurine feeding to man can increase the percentage of bile acids conjugated with taurine (3,4). If this is the case in guinea pigs, taurine-fed guinea pigs may be a useful model system for comparing the effects of glycine- and taurine-conjugated bile acids on the biosynthesis of bile acids.

This investigation deals with the effects of dietary taurine in guinea pigs on the conjugation pattern, composition, pool size, half-life, fractional turnover and synthetic rate of bile acids, as well as the specific activity of hepatic cholesterol 7α-hydroxylase and the level of cholesterol in both liver and serum.

MATERIALS AND METHODS

Animals and Diet

Male guinea pigs weighing 200-300 g (control, 258 ± 40 g; test animals, 256 ± 36 g) were used. The animals were kept in individual metabolic cages and were fed a stock diet consisting of guinea pig chow pellets which were purchased from Oriental Kobo Co. Cholesterol was not present in the diet as shown by gas liquid chromatography (GLC). For test animals, taurine was added to drinking water at the level of 0.5%. Taurine uptake was calculated from the water volume consumed every day. Daily taurine uptake was 200-300 mg in each animal. Fifty percent of controls and taurine-fed animals were killed on the 10th day and the gall bladders were excised to determine the glycine-taurine (G:T) ratio of bile acids. Food intake and body weights were measured every day. The 2 groups of animals gained weight at the same rate. At the end of the experiment period (4 wk), the 2 groups of animals were fasted for 24 hr and anesthetized with ether. Blood was taken from the neck vein to determine plasma cholesterol. The contents of the gall bladder, small intestine, caecum and large intestine were removed to determine the composition and pool size of bile acids. The liver was used to determine the hepatic cholesterol 7α-hydroxylase activity and the level of cholesterol concentration.

Bile Acid Turnover

For the studies of the half-life, fractional turnover and synthetic rate of bile acids, chenodeoxycholic acid-24-^{14}C (CDC-^{14}C) (19.3 mCi/mmol, Daiichi Chemical Co., Tokyo)

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was administered to the controls and the animals were fed taurine on the 13th day of the experiment. The CDC-^{14}C was dissolved in 25% aqueous ethanol. Each guinea pig was injected intraperitoneally with 0.5 ml (10 μCi) of the solution. During the 14 days following the injection, feces were collected every day and lyophilized. Radioactivity in the feces was measured.

Isolation and Quantitation of Bile Acids

Correction for losses during the procedures was made using taurochenodeoxycholate-24-^{14}C or glycochenodeoxycholate-24-^{14}C as an internal recovery standard. These labeled conjugates were synthesized from CDC-^{14}C according to the method described previously (5).

Gall bladder bile was extracted with 10 vol of ethanol at room temp, and the solvent was evaporated to dryness. When examined by thin layer chromatography (TLC), the residue consisted mainly of glycine- and taurine-conjugated bile acids, and little or no unconjugated and sulfated bile acid was detected. The solvent system for TLC were chloroform/methanol/acetic acid/water (65:20:10:5,v/v), n-butanol/acetic acid/water (85:10:5, v/v) and benzene/isopropanol/acetic acid (30:10:1,v/v). For the separation of glycine and taurine conjugates, the residue was dissolved in 15 ml of water, acidified to pH 1 with 4N HCl, and extracted with ether (10 ml x 2) and with ethyl acetate (10 ml x 2). The aqueous phase contained taurine conjugates, whereas the combined organic extracts contained glycine conjugates, which were re-extracted with two 10 ml portions of 2% NaOH solution. Recovery experiments using glycochenodeoxycholate-24-^{14}C and taurochenodeoxycholate-24-^{14}C resulted in 98-107% and 98-100 %, for glycine and taurine conjugates indicating the separation of glycine and taurine conjugates was essentially complete. Quantitation of individual bile acids in the glycine and taurine fractions was carried out by GLC after hydrolysis, methylation and silylation. The aqueous solution containing glycine or taurine conjugates was concentrated to 20 ml of vol, and mixed with 1 ml of 11N NaOH. The solution was heated in a sealed tube for 3 hr at 15 lb of pressure. The hydrolysate was diluted with water, acidified and extracted with ether (10 ml x 3). The combined ether extracts were washed with water until the washings became neutral, dried over anhydrous Na_2SO_4 and the solvent was evaporated to dryness. The residue was methylated with diazomethane and then silylated with a mixture of hexamethyldisilazane/trimethylchlorosilane/ pyridine (20:1:50) at room temp for 2 hr. As an internal GLC standard, 5α-cholestane was added, and the reaction mixture was subjected to GLC. The amount of glycine- or taurine-conjugated bile acids was estimated by the sum of the amounts of individual bile acids.

The small intestine content was extracted with hot ethanol for 6 hr. An aliquot of the extract thus obtained was hydrolyzed with 2N NaOH for 3 hr at 110 C. The bile acids were obtained by extracting with ether after acidifying the hydrolysate with 4 N HCl. Quantitation of individual bile acids was carried out by GLC after methylation and silylation. In order to check for sulfated bile acids, another aliquot of the extract was solvolyzed according to the method described previously (6), and then processed for GLC analysis. The amounts of individual bile acids measured by this procedure were identical to those of the bile acids measured by the procedure without solvolysis. These results indicated little or no sulfated bile acid was present in the small intestine content.

The caecum and large intestine contents were assayed using a modified Cohen et al. method (7). The contents were extracted with 80 ml of ethanol in a Soxhlet apparatus for 6 hr. The ethanol extract was concentrated to 20 ml of volume, mixed with 2 ml of 11N NaOH and refluxed for 1 hr in a water bath. The hydrolysate was diluted with 10 ml of water and extracted 3 times with 50 ml of petroleum ether to remove the neutral sterols (8). The aqueous phase containing the acidic steroids was evaporated to remove the ethanol. The volume was brought to 20 ml with water and 2 ml of 11N NaOH was added. The solution was autoclaved for 3 hr at 15 lb of pressure. Extraction of the bile acids from the hydrolysate was carried out as described above for the bile acids of small intestine content. Quantitation of individual bile acids was carried out by GLC of the methyl ester-trimethylsilyl ether derivatives.

GLC

All GLC analyses were carried out as previously described (9) using a Shimadzu GC-6A gas chromatograph. Identification of individual bile acids was made by comparison of their retention times with authentic standards on 4 different columns, 3% QF-1, 0.18% Poly I-110, 2% OV-1, and 3% OV-17. Quantitation of major bile acids was carried out on a 3% OV-17 column.

Measurement of Radioactivity

Radioactivity was measured using a toluene-

based cocktail in a Packard Tri-carb Model 3320 liquid scintillation spectrometer. Suitable corrections were made for background and quenching. Samples of ground feces were combusted in a Packard Model 306 biological material oxidizer. The resultant $^{14}CO_2$ was trapped in Oxisorb CO_2® (NEN Corp.) and counted with the toluene-based cocktail.

Assay of Hepatic Cholesterol 7α-Hydroxylase Activity

The specific activity (sp act) of hepatic microsomal cholesterol 7α-hydroxylase was determined according to Nicolau et al. (10). The assay system contained an NADPH-generating system, cholesterol-4-^{14}C (5 x 10^5 dpm), unlabeled cholesterol and microsomal protein (200-250 μg).

Liver and Plasma Cholesterol Concentration

Liver lipids were extracted with a mixture of $CHCl_3$/methanol (2:1) using the Folch et al. method (11). Aliquots of the extract were applied to a thin-layer plate (Silica Gel G, 20 cm x 20 cm x 0.5 mm). The plate was developed using petroleum ether/ether/acetic acid (90:10:1, v/v) and sprayed with a 10% solution of phosphomolybdic acid in ethanol and heated at 110 C for 5 min. The densities of spots resulting from free cholesterol and cholesteryl ester were measured by direct densitometry using a dual-wave-length chromatoscanner. The cholesterol standard was run simultaneously on the plate for correction. A detailed description for the procedure has been reported elsewhere (12).

The plasma cholesterol concentration was determined similarly except that aliquots of plasma were directly applied to a thin-layer plate.

RESULTS

Change of G:T Ratio

The oral administration of taurine (daily 200-300 mg) for 10 days increased 6-fold the taurine-conjugated bile acids in the gall bladder bile. The G:T ratio shifted from 3.95 to 0.19 by taurine feeding (Table I). The results are consistent with those in man (3).

Bile Acids in the Gall Bladder Bile and in the Contents of Small Intestine, Caecum and Large Intestine

Table I shows the composition of bile acids in the gall bladder bile and in the contents of small intestine, caecum and large intestine. The major bile acids in the gall bladder in control and taurine-fed animals were CDC, 7-ketolithocholic acid and ursodeoxycholic acid. There

TABLE I

G:T Ratio and Bile Acid Pool in Control and Taurine-Fed Guinea Pigs[a]

	Control animals[b]						Experimental animals[c]					
	G:T ratio	CDC	UDC	7-Keto	LCA	Total	G:T ratio	CDC	UDC	7-Keto	LCA	Total
Gall bladder	3.95 ± 1.19	4.12 ± 2.41	0.47 ± 0.26	3.26 ± 0.84	0.02 ± 0.01	7.87 ± 2.83	0.19[d] ± 0.11	3.65 ± 2.63	0.46 ± 0.11	4.74 ± 2.20	0.02 ± 0.01	8.87 ± 1.77
Small intestine		1.99 ± 0.85	0.15 ± 0.05	1.83 ± 0.60	0.02 ± 0.01	3.99 ± 1.48		1.06 ± 0.61	0.13 ± 0.04	0.99 ± 0.34	0.02 ± 0.01	2.20 ± 0.34
Caecum and large intestine		0.46 ± 0.19	0.03 ± 0.01	1.03 ± 0.16	1.67 ± 0.35	3.19 ± 0.68		0.47 ± 0.22	0.03 ± 0.01	1.12 ± 0.28	2.38[e] ± 0.19	4.00 ± 0.59
Pool size		6.57 ± 2.27	0.65 ± 0.23	6.12 ± 0.28	1.71 ± 0.35	15.05 ± 2.40		5.18 ± 3.01	0.62 ± 0.12	6.84 ± 2.61	2.43[e] ± 0.19	15.06 ± 1.27

[a]Data (mean ± SD) are represented as mg/500 g body weight, except G:T ratio. CDC = chenodeoxycholic; UDC = ursodeoxycholic; 7-Keto = 7-ketolithocholic; LCA = lithocholic acid.
[b]Number of animals = 3; mean body weight = 376 ± 40 g.
[c]Number of animals = 4; mean body weight = 365 ± 18 g.
[d]Significantly different from control, $p < 0.001$.
[e]$p < 0.02$.

was some lithocholic acid in the gall bladder bile in the 2 groups. The bile acid composition is similar to that reported previously (13). Taurine feeding for 4 wk did not change the bile acid composition in the gall bladder bile.

The major bile acid in both groups was lithocholic acid in the contents of the caecum and large intestine. The amount (mg/500 g body weight) of this secondary bile acid increased from 1.67 to 2.38 ($p < 0.02$) by taurine feeding.

Bile Acid Pool Size

The total amount of bile acids in the gall bladder, small intestine, caecum and large intestine can account for more than 90% of the bile acid pool in guinea pigs that are fasted for 24 hr (14). The pool size did not change by taurine feeding for 4 wk, although the total amount of lithocholic acid increased about 40% from 1.71 to 2.43 mg/500 g body weight (Table I).

Bile Acid Kinetics

Guinea pigs have only CDC and its metabolites, 7-ketolithocholic acid, ursodeoxycholic acid and lithocholic acid (14). Thus, it is possible to determine the metabolism of total bile acid pool by administering CDC-24-^{14}C. The turnover rate of CDC was calculated according to the Lindstedt and Norman method (15). In Figure 1, $-\log(1-U^t/U^{max})$ was plotted against time, where U^{max} = the total activity (dpm) recovered in feces, and U^t = the activity (dpm) of the fecal excretion accumulated up to a given time t. The dotted horizontal line cuts the excretion curves at the half-life. Figure 1 shows the half-life of CDC in guinea pigs fed taurine is significantly shorter than in controls ($p < 0.01$). The fractional turnover rate (day^{-1}), which is calculated from the half-life and the slope of the excretion curve in Figure 1, was greater in the taurine-fed group than in the control ($p < 0.05$). The synthetic rate of bile acids was the product of the fractional turnover rate (day^{-1}) and bile acid pool size (mg). The synthetic rate increased by 70% after taurine feeding (Table II).

Hepatic Cholesterol 7α-Hydroxylase Activity

The levels of hepatic microsomal 7α-hydroxylase activity in control and taurine-fed groups are shown in Table II. The optimal conditions for the assay system in guinea pigs were similar to those for man or for the rat liver enzyme (10,16). Each value of enzyme activity was corrected for nonenzymatic cholesterol oxidation by subtracting the values obtained for the boiled enzyme control. The enzyme activity in guinea pigs fed taurine was ca. 3-fold that in controls.

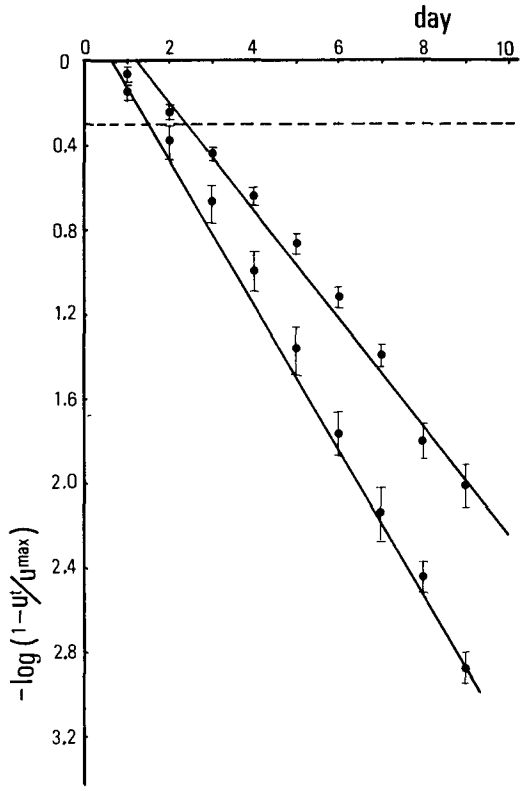

FIG. 1. The rate of elimination of radioactivity from bile acid pool. Each point represents the mean ± SD. The dotted horizontal line intersects the rate lines for each plot at the half-life. Linear regression equations and correlation coefficients (r) are as follows: control (n = 3), y = (0.264 ± 0.016) x - (0.333 ± 0.058), r = 0.984 ± 0.008; taurine-fed animals (n = 4), y = (0.336 ± 0.075)x - (0.227 ± 0.130), r = 0.969 ± 0.027.

Cholesterol in Plasma and Liver

Free and esterified cholesterol were separated by TLC and measured by direct densitometry (see Methods). The results are shown in Table II. There were substantial variations in the levels of the plasma cholesterol. The difference in mean values was not significant statistically in the 2 groups.

DISCUSSION

In guinea pigs, glycine-conjugated bile acids predominate over taurine-conjugated bile acids (G:T ratio, ca. 4:1). Recently, Vessey demonstrated that guinea pig liver contains a very low concentration of taurine and a high concentra-

TABLE II

Bile Acid Metabolism in Guinea Pigs[a]

	Control (n=3)	Taurine-fed animals (n=4)	t-test
Kinetics of bile acids			
Half-life (day)	2.39 ± 0	1.48 ± 0.35	$p < 0.01$
Fractional turnover (day^{-1})	0.29 ± 0.01	0.49 ± 0.13	$p < 0.05$
Synthetic rate (mg/day/500 g body weight)	4.28 ± 0.54	7.27 ± 1.74	$p < 0.05$
Cholesterol 7α-hydroxylase activity (7α-hydroxycholesterol dpm/mg protein/20 min)	1.29 ± 0.14	2.98 ± 0.50	$p < 0.01$
Liver cholesterol (mg/g wet liver)			
Free	2.05 ± 0.05	1.95 ± 0.24	NS[b]
Ester	1.43 ± 0.16	1.44 ± 0.12	NS
Plasma cholesterol (mg/dl)			
Free	8.10 ± 2.10	6.40 ± 0.80	NS
Ester	37.70 ± 11.3	49.70 ± 18.1	NS

[a]Data are expressed as mean ± standard deviation.

[b]NS = not significant.

tion of glycine. Furthermore, the reaction of choloyl-CoA with taurine and glycine, when catalyzed by soluble fraction from guinea pig liver, has a high affinity for taurine and a poor affinity for glycine (17). Based on these findings, Vessey suggested the predominance of glycine conjugation may be the result of a low taurine pool available for bile acid conjugation. This hypothesis is further supported by our observation that the oral administration of taurine to guinea pigs for 10 days significantly increased ($p < 0.001$) taurine-conjugated bile acids and reversed the G:T ratio. Thus, it is possible to study the effects of changes in the conjugation pattern on the bile acid metabolism by comparing the bile acid kinetics between taurine-fed animals and controls.

The half-life of CDC was reduced from 2.39 to 1.48 day by taurine feeding, suggesting the half-life of taurochenodeoxycholate (TCDC) is shorter than that of glycochenodeoxycholate (GCDC) in guinea pigs. The synthetic rate of bile acids (mg/day/500 g body weight) increased from 4.28 to 7.27 by taurine feeding. These results were confirmed by an unpublished observation that the fecal excretion (mg/day/500 g body weight) of bile acids measured by GLC increased ca. 20% from 5.28 to 6.34 by taurine feeding. The synthetic rate was augmented by taurine feeding, which is consistent with the data that the hepatic cholesterol 7α-hydroxylase activity increased 2 to 3-fold by taurine feeding.

In the ileum, bile acids are extensively absorbed by an active transport system (18) and return to the liver via the portal vein. The relatively small losses of bile acids into feces are replaced by further hepatic synthesis which is regulated by negative feedback control to keep the size of bile acid pool constant. Therefore, our results are reasonable since the half-life of CDC got shorter by ca. 40%, the synthetic rate of bile acids increased ca. 70% and the hepatic cholesterol 7α-hydroxylase activity was augmented 2.4-fold by taurine feeding.

The change in the G:T ratio of bile acids may influence cholesterol absorption from the gut, which may affect the hepatic bile acid synthesis. In the experiment by Gallo-Torres et al. (19), GCDC was more effective than TCDC in cholesterol absorption in the rat gut, which suggests taurine administration might be disadvantageous to cholesterol absorption in the gut, since taurine feeding increases TCDC instead of GCDC in guinea pigs. However, this does not explain why the synthetic rate of bile acids was enhanced by taurine feeding.

Our results suggest oral administration of taurine increased TCDC percentage of the bile acid pool, augmented the turnover rate of CDC and increased hepatic bile acid synthesis to keep the size of bile acid pool constant.

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Effects of Diet and High Density Lipoprotein Subfractions on the Removal of Cellular Cholesterol

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ABSTRACT

The effects of isocaloric substitutions of dietary polyunsaturated and saturated fat on the composition and function of plasma high density lipoproteins (HDLs) were studied in 3 normal subjects who were fed saturate-rich and polyunsaturate-rich diet programs. Compared to the saturated diets (P/S = 0.4), polyunsaturated fat diets (P/S = 4 or 2) reduced both plasma cholesterol and triglyceride levels. In 2 of the subjects, HDL cholesterol concentrations increased with polyunsaturated fat feeding; the third subject had no change in HDL cholesterol. Dietary polyunsaturated fat caused a reduction in HDL fatty acyl content of oleate and an increase in linoleate. To determine whether the altered composition affected the removal of cell membrane cholesterol, HDL and their subfractions, HDL_2 and HDL_3, which were isolated from each of the diets, were incubated with Ehrlich ascites cells in vitro. The cells were prelabeled with [^{3}H] cholesterol, and the release of labeled cholesterol from the cells into the medium containing the various HDL fractions was determined. HDL, irrespective of the type of dietary fat, caused a release of [^{3}H] cholesterol from the cells into the medium. The amount of [^{3}H] cholesterol recovered in the medium was dependent on the absolute concentration of HDL cholesterol added to the cells and was independent of the type of diet. These results indicate that HDL facilitates the removal of cholesterol from cells, but that the amount and rate of removal are independent of the changes in HDL composition that can be obtained by dietary perturbations.

INTRODUCTION

Plasma low density lipoproteins (LDLs) and high density lipoproteins (HDLs) are the major mediators of cholesterol transport in the body (1). The donors of cellular cholesterol in man are LDLs. The uptake of LDL by extrahepatic cells is mediated by specific membrane receptors (2). The function of HDL is to facilitate the egress of cholesterol from cells (3). Glomset (4) has proposed that the esterification of cholesterol by plasma lecithin/cholesterol acyltransferase (LCAT) facilitates the removal of cholesterol from cells by HDL. In addition, studies by Bates and Rothblat (5,6) and Stein et al. (7,8) suggest that even in the absence of LCAT activity, HDLs function to remove free cholesterol from cells. Whether the absolute amount of HDL or the composition of HDL determines the egress of cellular cholesterol is not known. Recent studies (9-12) have shown that polyunsaturated fat feeding in man alters the fatty acid composition of HDL lipids such that there is an increase in linoleic acid and a decrease in palmitic and oleic acid content. Changes in HDL composition, induced by diet, have been shown by Shepherd et al. (12) to have no effect on the fractional catabolic rate of catabolism of their major protein, apo A-I. The effects of altered HDL composition on the removal of cholesterol from cells have not been reported. This investigation was undertaken in order to determine whether changes in the composition of HDL that are produced by isocaloric changes in dietary polyunsaturates and saturates alter either the absolute amount or the rate by which HDL facilitates the removal of cholesterol from cells.

MATERIALS AND METHODS

The subjects for this study were 3 normal healthy white males with normal lipoprotein patterns and plasma lipid concentrations. Subject 1 (39 years of age), subject 2 (24 years of age) and subject 3 (20 years of age) were on no medications and maintained constant body weight throughout the study. The subjects were investigated in the University of Cincinnati General Clinical Research Center and received only food prepared in the Research Center metabolic kitchen. Cholesterol intake was 400 mg/day and the calorie distribution was 40%, 40% and 20% as carbohydrate, fat and protein, respectively. In the first phase of the study, the polyunsaturated/saturated (P/S) fat ratio was 4.0 for subjects 1 and 3, and 2.0 for subject 2. In the second phase, each subject received a P/S fat ratio of 0.4. After 2 wk of each respective diet, 600 ml of plasma were taken from each subject by plasmaphoresis; final concentrations of 0.01% ethylene diaminetetraacetic acid, 0.01% sodium azide and 10^{-3}M phenylmethyl

sulfonyl fluoride were immediately added to the plasma and lipoproteins were isolated within 1 wk.

Lipoproteins were isolated by ultracentrifugation in salt solutions of KBr. HDLs were isolated between d 1.063-1.210; HDL_2 (d 1.063-1.120); and HDL_3 (d 1.120-1.210). The isolated lipoproteins were refloated at their highest densities, dialyzed against a buffer containing 10 mM Tris-HCl, pH 7.4, 0.9% NaCl and 0.01% sodium azide. HDL or its subfractions formed no detectable precipitant lines on Ouchterlony plates with antisera to either LDLs or to albumin. Plasma cholesterol and triglyceride were measured by autoanalyzer II methodologies as described for the Lipid Research Clinic's Methodology (13). Protein concentrations were determined by the Lowry et al. method (14), phospholipid by the Bartlett method (15), cholesterol, both free and esterified, by enzymic procedures (Mannheim Boehringer) (16), and triglyceride by the Autoanalyzer LRC procedure (13). Lipoprotein lipids were extracted by the Folch procedure (17). Total saponifiable lipoprotein fatty acids were determined by gas chromatography (GC) (18).

The Ehrlich ascites cells were isolated from male CBA mice 11-14 days following transplantation (19). Twelve hr before harvesting the cells, the mice were injected intraperitonally with 25 μCi each of 7 (n) [^{3}H] cholesterol, 9.5 Ci/mmol (Amersham, England). [^{3}H]-Cholesterol was injected as microcrystalline suspension in 0.20 ml of 0.9% NaCl. The cells were removed, suspended in Krebs-Henseleit phosphate buffer, pH 7.4, washed 5 times with Krebs-Henseleit buffer containing 2% fatty acid-free bovine serum albumin in order to remove any unbound cholesterol, and were finally diluted with the phosphate buffer to give 10^8 cells/ml. The range of radioactivity for 10 different preparations was 1.2-2.0 x 10^5 cpm/10^8 cells.

In all experiments, ascites cells were incubated in a total volume of 1.0 ml with various HDL fractions. Unless indicated differently, all experiments were performed at 37 C in a shaking water bath. At appropriate incubation times, the cells were rapidly cooled and pelleted at 4 C by centrifugation (2,000 x g) for 15 min. [^{3}H] Cholesterol was determined in the medium by liquid scintillation counting, and the total cholesterol (free and esterified) content was measured enzymatically (16).

RESULTS

Plasma Lipids and Lipoproteins

The effects of dietary polyunsaturated or saturated fats on total plasma cholesterol and triglycerides and HDL-cholesterol are shown in Table I. Compared to the saturated fat diets, the polyunsaturated fat diets caused a decrease in both plasma cholesterol and triglycerides in each of the subjects, findings which are consistent with previous studies (9-12). In contrast, HDL-cholesterol levels were not reduced by polyunsaturated fat feeding. Subjects 1 and 2 had increased HDL-cholesterol on the polyunsaturated fat diet as compared to the saturated one; the concentration of HDL-cholesterol in subject 3 did not change. HDLs were isolated from each subject and the total fatty acid composition was determined (Table II). In general, the results are similar to those reported previously (9-12). Ingestion of poly-

TABLE I

Effects of Dietary Fat on Plasma Lipids[a]

Subject	Diet P/S ratio	Plasma cholesterol mg/100 ml	Plasma triglyceride mg/100 ml	HDL cholesterol mg/100 ml
1	0.4	163	97	49
	4.0	144	80	53
	% change	↓13%	↓21%	↑7%
2	0.4	158	94	43
	2.0	148	53	46
	% change	↓7%	↓44%	↑6%
3	0.4	125	61	52
	4.0	114	58	52
	% change	↓10%	↓5%	N.C.

[a]The numbers represent the average of duplicate analyses of plasma lipids after 2 wk of each respective diet. In all determinations, the duplicates were always within ± 3% of each other.

TABLE II

Changes in High Density Lipoprotein Fatty Acids Induced by Altered Dietary Fat Saturation[a]

Fatty acids	Subject 1		Subject 2		Subject 3	
	S	P	S	P	S	P
14:1	0.3	0.2	0.5	0.1	0.4	0.2
16:1	1.1	1.0	1.6	0.9	1.7	1.5
18:0	7.0	6.7	7.1	7.8	10.3	8.6
18:1	16.9	11.4	15.0	12.4	19.9	12.2
18:2	37.2	40.7	34.9	40.5	28.6	36.7
<18:2-20:4	16.7	23.8	23.8	17.3	15.7	19.0

[a]The numbers represent the average percent of duplicate analysis of each fatty acid of total HDL (d 1.063–1.210) lipids on saturated (S) or polyunsaturated (P) diets (Fig. 1).

unsaturated fat caused an increase in the percentage of 18:2 and a reduction in 18:1 when compared to the saturated diet. The effects of dietary fat saturation on the lipid and protein composition of HDL and its subfractions are shown in Table III. It was not possible to determine any significant differences between the lipid and/or protein composition of the various HDL fractions collected during the 2 dietary periods.

Cholesterol Exchange with Ascites Cells

To determine the effects of HDL isolated from the 2 types of diets on the removal of cholesterol, Ehrlich ascites cells were incubated with the various HDL preparations. The ascites cells used in these studies were prelabeled with [^{3}H]cholesterol 12 hr prior to their removal from the mice. The washed cells contained ca. 87% of the radioactivity as cholesterol and 13% as cholesteryl esters.

In the first experiment, total HDL (d 1.063 -1.210) from the saturated diet of subject 1 was incubated with labeled cells in order to determine the kinetics of release of [^{3}H]cholesterol. The addition of HDL (90 μg free cholesterol) resulted in a progressive release of [^{3}H]-cholesterol into the incubation medium and approached a maximum value of 22% after 2 hr (Fig. 1).

In the next experiment, we attempted to determine whether HDL isolated from the different dietary manipulations affected the amount of [^{3}H]cholesterol removed from cells. Increasing amounts of total HDL (30-250 μg free cholesterol) were incubated with 2 x 10^7 cells, and the amount of [^{3}H]cholesterol removed in 2 hr of incubation was determined. As shown in Figure 2, total HDL from either the polyunsaturated or the saturated dietary periods of subjects 1 and 2 caused the same amount of cholesterol to be released. Identical results were obtained with the HDL from subject 3. The effects of ascites cell concentration on the isotopic equilibration of cellular, labeled cholesterol with HDL cholesterol in the medium was also determined. In these experiments, a fixed amount (30 μg free cholesterol) of each HDL fraction was incubated with increasing amounts of labeled cells. As shown in Figure 3, there was a progressive increase in the

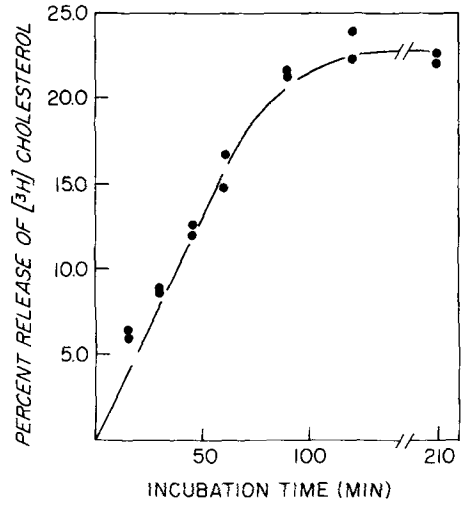

FIG. 1. Effect of incubation time on the removal of [^{3}H]cholesterol from ascites cells. Two x 10^7 ascites cells (2.0 x 10^5 cpm/10^8 cells) were incubated at 37 C with 90 μg of HDL free cholesterol (subject 1, saturated diet) in a final volume of 1.0 ml of Krebs-Henseleit buffer containing 2 mg/ml glucose. At the indicated times, samples were removed, cells pelleted by centrifugation and the percent [^{3}H]cholesterol extracted into the medium determined as described in Materials and Methods. The percent released into the medium was calculated taking the total radioactivity in the cell as 100% and subtracting the counts released in the absence of added HDL from each experimental value (1-2%). Each value represents individual experiments.

TABLE III

Effect of Dietary Fat Saturation on HDL Composition[a]

Subject	HDL fraction	Total cholesterol		Triglyceride		Phospholipid		Protein	
		S (%)	P (%)	S (%)	P (%)	S (%)	P (%)	S (%)	P (%)
1	HDL	12.6	16.7	2.7	5.3	34.0	33.7	50.7	44.3
	HDL_2	15.5	17.5	3.4	5.4	38.2	39.7	42.0	37.4
	HDL_3	12.8	14.0	2.6	3.9	30.4	33.0	54.2	49.1
2	HDL	14.6	17.1	5.0	1.9	32.8	33.0	47.6	48.0
	HDL_2	14.1	15.4	4.0	5.8	27.4	30.8	54.0	47.7
	HDL_3	13.0	11.7	3.4	4.9	25.9	24.1	57.1	59.9
3	HDL	19.2	18.7	6.9	4.3	25.9	26.5	49.9	50.5
	HDL_2	21.7	17.9	7.9	5.3	28.3	25.7	49.1	47.1
	HDL_3	12.5	19.4	6.7	3.7	23.9	25.9	49.7	57.7

[a]The values represent the weight percent of each constituent.

specific activity (sp act) (cpm [^{3}H] cholesterol/ μg medium cholesterol) as increasing amounts of cells were added; the sp act was the same for total HDL from either the saturated or polyunsaturated diets.

Finally, each of the HDL subfractions were tested for their effects on the removal of [^{3}H]-cholesterol from ascites cells. As shown in Table IV, there was essentially no difference between HDL_2 and HDL_3 in removing [^{3}H]-cholesterol. Furthermore, the type of dietary fat had little effect on the amount of [^{3}H]-cholesterol removed. There were some differences among subjects, but these resulted from different cell preparations.

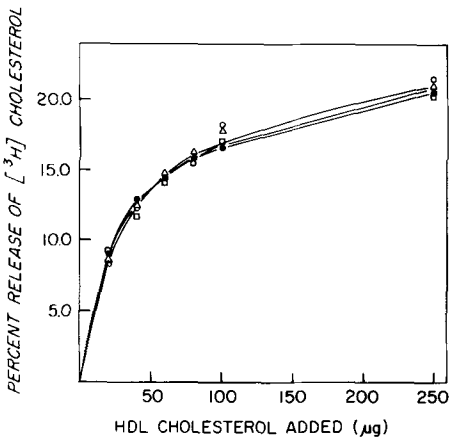

FIG. 2. Effect of high density lipoprotein cholesterol concentration on the removal of [^{3}H] cholesterol from ascites cells. Two x 10^7 cells (1.2 x 10^5 cpm/10^8 cells) were incubated with various amounts of HDL (measured as free cholesterol) in a final volume of 1.0 ml of Krebs-Henseleit buffer containing 2 mg/ml glucose. After 2 hr at 37 C, cells were pelleted by centrifugation and the percent [^{3}H] cholesterol released into the medium determined as described in Figure 1. Symbols: (−●−●−) HDL, subject 1, polyunsaturated diet; (−○−○−) HDL, subject 2, polyunsaturated diet; (−△−△−) HDL, subject 1, saturated diet; (−□−□−) HDL, subject 2, saturated diet.

DISCUSSION

As expected from previous studies (9-12), isocaloric substitution of polyunsaturates for saturates reduced plasma cholesterol and triglyceride. The effects of dietary fat on HDL levels and composition are more controversial (9-12,20-23). In 4 normal subjects, Shepherd, et al. (12) recently reported HDL-cholesterol decreased 33% on diets with a P/S of 4.0 compared to diets with a P/S of 0.25. In another study (11) of a single subject with hyperlipoproteinemia (Type 11b lipoprotein phenotype), Shepherd et al. found that polyunsaturates had no effect on HDL-cholesterol concentration. In contrast, Farquhar and Sokolow (22) and Spritz and Michkel (23) have shown that polyunsaturated fat feeding caused an increased HDL-cholesterol. In addition, 2 controlled clinical studies (20,21) showed an increased HDL-cholesterol with polyunsaturated fat diets. In both of the studies of Shepherd et al. (11,12), polyunsaturates produced a decreased rate of synthesis of apo A-1, the major protein constituent of HDL.

Although the effects of dietary fat on HDL cholesterol may be controversial, it is generally agreed that polyunsaturated fat feeding alters HDL fatty acyl composition by increasing the amount of linoleate. To evaluate the possible effects of altered fatty acyl composition on the function of HDL, we have measured the removal of [^{3}H] cholesterol from ascites cells using the HDL isolated during the various dietary periods. There were no differences

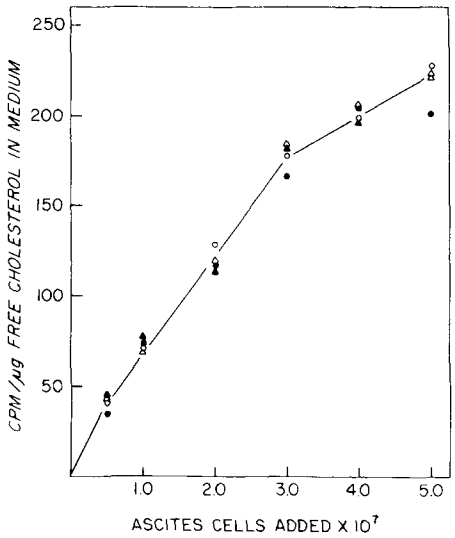

FIG. 3. Effect of ascites cell concentration on the activity of cholesterol in the medium. The indicated amounts of ascites cells (1.3 x 10^5 cpm/10^8 cells) were incubated with HDL (30 μg free cholesterol) in a final volume of 1.0 ml of Krebs-Henseleit buffer containing 2 mg/ml glucose. Afte 2 hr incubation, the cells were pelleted by centrifugation and the amount of [^{3}H] cholesterol in the medium determined. Total medium cholesterol was determined by enzymic methods (see Materials and Methods). The ratio (cpm/μg free cholesterol in medium) was determined by dividing the total radioactivity in the medium by the total free cholesterol. Each value represents the average of duplicate analyses. Symbols: (−●−●−) HDL, subject 1, polyunsaturated diet; (−○−○−) HDL, subject 1, saturated diet; (−▲−▲−) HDL, subject 2, polyunsaturated diet; (−△−△−) HDL, subject 2, saturated diet.

exhibited by either total HDL or the HDL subfractions in either the rate of [^{3}H] cholesterol removed or in the absolute amount of removal.

The results of these experiments in man are different from those reported in the rabbit and pig (24,25). Spritz (24) and Yeh et al. (25) found that the exchange of cholesterol from either very low density lipoproteins (VLDLs) or plasma was affected by the type of diet. Spritz (24) reported that VLDL isolated from rabbits fed safflower oil exchanged cholesterol significantly faster than cholesterol in VLDL from rabbits fed coconut oil. Similarly, Yeh et al. (25) found that the exchange of cholesterol between plasma and red blood cells from pigs fed hydrogenated soybean oil was slower than the exchange in the chow-fed animals. In addition, Bloj and Zilversmit (26), using a model system with cholesterol/phospholipid vesicles, demonstrated that the rate of cholesterol exchange between the vesicles and red

TABLE IV

Effect of HDL Subfractions on the Removal of [^{3}H] Cholesterol from Ascites Cells[a]

Subject	Lipoprotein	% Release of [^{3}H] cholesterol	
		Sat. diet	Polyunsat. diet
1	HDL_2	10.3	9.1
	HDL_3	10.0	8.9
2	HDL_2	6.0	5.9
	HDL_3	6.0	6.6
3	HDL_2	6.0	4.0
	HDL_3	5.8	5.6

[a]The numbers represent the average of duplicate analyses of the percent [^{3}H] cholesterol removed from ascites cells. Two times 10^7 cells (1.7 x 10^5 cpm/10^8 cells) were incubated at 37 C for 2 hr with the indicated HDL samples (30 μg free cholesterol each). Cells were pelleted by centrifugation and the percent [^{3}H]-cholesterol was removed determined as described in Figure 1.

blood cells was faster when the vesicles are composed of unsaturated phosphotidylcholines as compared with saturated ones. Jackson et al. (27) also reported that the net egress of cholesterol from ascites cells is affected by the type of phospholipid fatty acyl chains. The removal of cholesterol was increased with apoprotein/saturated phospholipids as compared to unsaturated phospholipids (27). Thus, while there is evidence that the rate of cholesterol exchange or the net efflux of cholesterol from cells can be controlled by the phospholipid composition, we have failed to demonstrate these differences using HDL isolated from plasma of humans fed either a polyunsaturate-rich or saturate-rich diet program. The most likely explanation for these insignificant differences is undoubtedly related to the diet which does not alter the lipoprotein sufficiently enough to have extremes in composition as that used in the animal studies.

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Isolation and Analysis of Age-Related Fluorescent Substances in Rat Testes

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ABSTRACT

Fluorescent substances were extracted from rat testicular tissue with 2,2-dimethoxypropane (DMP) and analyzed by 2-dimensional thin layer chromatography (TLC). One substance that accumulated with increasing age of the animals was isolated and analyzed quantitatively by spectrophotofluorometry using quinine sulfate as a standard. This substance, which was designated as an age-related fluorescent substance (ARFS), exhibited an excitation maximum at 355 nm and an emission maximum at 490 nm. Its fluorescence was quenched by metal chelators and at alkaline pH, indicating it contained a conjugated Schiff base structure. Quantitative analysis of this substance in the testes of rats 1, 2, 11 and 20 months of age showed that it increased linearly with age. The relation of this substance to aging also was indicated by its detection in animals of different ages fed diets of both low and high unsaturation.

INTRODUCTION

The formation of fluorescent substances as products of lipid oxidation has been well established from studies with model systems (1-3). Lipofuscin substances accumulate with increasing age in mammalian tissues and are believed to originate from in vivo oxidation (4-7). However, use of fluorescence measurements as an assay of in vivo oxidation is complicated by the presence of natural fluorescent compounds (8-10). Fletcher et al. (10) resolved this problem, in part, by subjecting the sample to ultraviolet light which destroys retinol, a major interfering substance, prior to analysis. Csallany et al. (9) found that retinol could be separated by Sephadex chromatography and developed a procedure for the analysis of fluorescent substances associated with aging based on prefractionation of chloroform/methanol tissue extracts by this technique. Another complication in the analysis of fluorescent substances associated with aging in animal tissues is their complexity (11,12). Trombly et al. (8) showed by silicic acid column chromatography that even with relatively simple model systems, a variety of fluorescence compounds are products of lipid oxidation and that testes of rats and humans contain at least 3 major fractions or families of fluorescent compounds. Studies of thin layer chromatography (TLC) analysis of the fluorescent products of lipid oxidation of model compounds, as well as the occurrence of lipofuscin substances in human tissues in this laboratory, also demonstrated the complexity of these substances (13,14). In these studies, 2-dimensional TLC revealed one fluorescent compound in particular that appeared to be related to aging inasmuch as it was found in much larger amounts in old animals than in young animals. This substance was designated as an age-related fluorescent substance (ARFS) because its presence in animal tissues also correlated well with histologic analysis of the aging pigment (14).

The isolation of this substance from testicular tissue and its formation in animals of different ages fed diets differing in fatty acid composition is reported here.

EXPERIMENTAL

Animals

Weanling male Sprague-Dawley rats obtained from ARS Sprague-Dawley, Madison, WI were housed on wire in individual cages with free access to water and food. The diets consisted of Purina Lab Chow supplemented with 10% by weight of corn oil or a mixture of corn and menhaden oil (3:7). In another experiment, animals were fed a semisynthetic fat-free diet supplemented with 5% hydrogenated coconut oil (HCO) or 5% safflower oil (SAFF) from weanling to 11 months of age. The composition of this diet and the fatty acid composition of the dietary fat supplements are shown in Tables I and II, respectively.

The testes of each animal were excised, washed with cold saline and stored at -70 C prior to being processed. Generally, no tissue was stored for more than 3 wk.

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Chemicals and Solvents

2,2-Dimethoxypropane (DMP), Eastman, Rochester, NY, was purified by distillation over KOH pellets. Chloroform and methanol used as solvents for TLC also were purified by distillation prior to use. Europium [*tris* (2,2,6,6-tetramethyl-3,5-heptanedionate)], [$Eu(thd)_3$] was obtained from Aldrich Chemical Co., Milwaukee, WI. Silica Gel H was obtained from Merck Ag., Darmstadt, Germany, and washed twice with chloroform-methanol (2:1, v/v) to remove fluorescent impurities.

TLC

Chromatoplates for TLC were prepared with a 0.3 mm coating of the Silica Gel H and activated by heating for 1 hr at 110 C. One-dimensional TLC was carried out with chloroform-methanol-acetic acid-water (95:5:1:0.3, v/v/v/v). Two-dimensional TLC was carried out with the same solvent in the first dimension and chloroform-methanol-ammonium hydroxide (90:10:1, v/v/v) in the second dimension. The fluorescent compounds were detected by viewing the plates under ultraviolet light; the spots were scraped into small test tubes and extracted with ethanol. Extraction was effected by thorough mixing on a vortex mixer for 1-2 minutes followed by centrifugation at 3000 rpm for 10 min.

Extraction of Tissue

The tissue (0.5 g), was placed into a 50 ml centrifuge tube and extracted with 20 ml of DMP containing 20 μl of acetic acid by homogenization with a Polytron homogenizer for 1 min at half speed. The mixture was allowed to stand for 1 hr with occasional mixing and centrifuged at 3000 rpm for 10 min. The DMP layer was separated and the residue rehomogenized with 10 ml of DMP containing 10 μl of acetic acid for 30 sec. The 2 DMP extracts were combined, reduced in volume under reduced pressure on a rotary evaporator and finally taken to dryness under an atmosphere of nitrogen, then dissolved in 2 ml of chloroform.

Chloroform-methanol extractions of testicular tissue were carried out as described by Fletcher et al. (10). Fatty acid composition was determined by gas liquid chromatography (GLC) of methyl esters as previously described (15,16).

Fluorescence Measurements

Excitation and emission spectra were measured with an Aminco-Bowman spectrophotofluorometer, model D223-62155 with entrance and exist slits of 3 mm and photomultiplier slit of 5 mm (American Instrument Co., Silver Springs, MD). Quinine sulfate, 1 μl/ml in 0.1 N H_2SO_4, was used as the fluorescence standard which gave a relative fluorescence intensity of 24 at a meter multiplier setting of 0.01 and a sensitivity setting of 50.

The effect of pH and metal chelation using $Eu(thd)_3$ was determined in methanol as described by Malshet et al. (17).

TABLE I

Diet Composition, % by wt

Casein vitamin test	22.5
Wesson salt mixture[b]	4.0
Vitamin mix[c]	1.0
Sucrose	56.0
Choline mix[a]	1.0
Cellulose alphacel	10.5
Fat	5.0

[a]Choline mix consists of 22% choline dihydrogen citrate in vitamin test casein.

[b]Wesson salt mixture does not contain zinc or manganese, hence these elements are added to the mix as follows: 0.60 g of $ZnCl_2$ and 0.90 g of $MnSO_4.H_2O$ per 200 g of salt mixture.

[c]Fat and vitamins A, D and E are mixed into the diet daily and stored at 0 C overnight. Vitamin D_2, 5.0 mg; retinol acetate, 6.9 mg; α-tocopherol acetate, 300 mg per kg of diet. Vitamin mix (grams): thiamine HCl 2.5, riboflavin 2.5, nicotinic acid 9.0, calcium pantothenate 9.0, pyridoxine HCl 2.0, cyanocobalmin (B_{12}) 4.0, *p*-amino benzoic acid 7.5, folic acid 0.1, biotin 0.02, *meso*-inositol 20.0 mendione (vitamin K) 0.5, vitamin test casein 943.0.

TABLE II

Fatty Acid Composition of Dietary Fats, % wt[a]

	SAFF.	HCO	Corn	Corn-Menh. 3:7
10:0	---	2	---	---
12:0	---	7	---	---
14:0	---	36	---	8
16:0	10	19	11	19
16:1	---	---	---	11
18:0	4	28	2	4
18:1	13	8	25	17
18:2	73	---	61	20
20:0	---	---	1	2
20:5	---	---	---	12
22:6	---	---	---	7

[a]SAFF = Safflower oil; HCO = hydrogenated coconut oil; CO = corn oil; Corn-Menh. = Corn-Menhaden oil mixture, 3:7.

RESULTS AND DISCUSSION

Lipofuscin substances are generally ex-

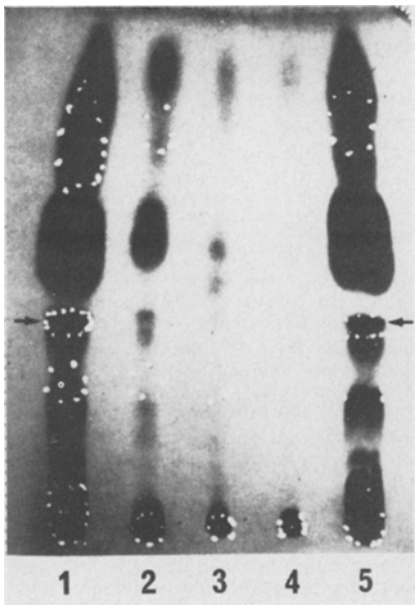

FIG. 1. Thin layer chromatographic analysis of testicular tissue extracts in Silica Gel H. Solvent chloroform-methanol-acetic acid-water (95:5:1:0.3, v/v/v/v). 1 = first extraction with DMP; 2 = second extraction with DMP; 3 = third extraction with DMP; 4 = extraction of residue of DMP extractions with chloroform-methanol (2:1, v/v); 5 = extraction with chloroform-methanol. Dotted spots = fluorescent components; spots were visualized by charring otherwise. The arrow points to the age-related fluorescent substances (ARFS).

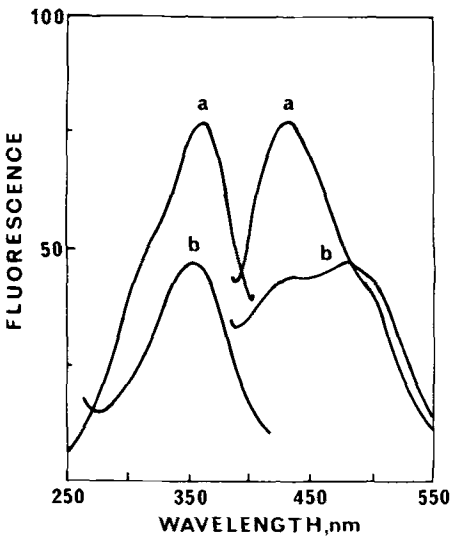

FIG. 2. Fluorescence spectra of the total extract of 0.2 g of testicular tissue obtained by (a) DMP and (b) chloroform-methanol (2:1, v/v) extractions, meter multiplier 0.01, sensitivity 50.

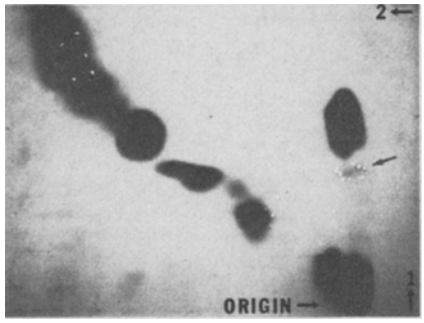

FIG. 3. Two-dimensional TLC of DMP extract of rat testicular tissue showing the separation of the ARFS (arrow). Solvent chloroform-methanol-acetic acid-water (95:5:1:0.3, v/v/v/v) first dimension, and chloroform-methanol-ammonium hydroxide (90:10:1, v/v/v) in the second dimension.

tracted from animal tissues with chloroform-methanol (2:1, v/v) which extracts a variety of other fluorescent compounds (9,10). Water-soluble fluorescent compounds can be removed by washing the organic extract with water, but these processes are generally complicated by troublesome emulsions. The course of the extraction of lipid and fluorescent substances with DMP is demonstrated by TLC analysis in Figure 1. These analyses showed that most of the lipid and the organic soluble fluorescent substances were extracted in the first DMP extraction. Except for a small amount of highly polar material that remained at the origin in the analyses in Figure 1, 3 extractions with DMP gave essentially a complete recovery of the lipid and fluorescent substances inasmuch as little material was recovered by a further extraction with chloroform-methanol. A comparison of No. 1 and No. 5 in Figure 1 also showed that DMP was essentially as efficient as chloroform-methanol for the extraction of animal tissues. In fact, the fluorescence spectra in Figure 2 indicate more fluorescent material was extracted by DMP than chloroform-methanol, particularly substances with a maximum at 440 nm.

The substance identified as an ARFS in previous work (14) was completely separated from other fluorescent compounds, as well as lipids, by 2-dimensional TLC as shown in Figure 3. None of the ARFS were detected in the testes of young animals, up to ca. 8 wk of age, but it accumulated thereafter in linear fasion with increasing age as illustrated in Figure 4A.

The ARFS contained a conjugated Schiff base as indicated by its fluorescence spectrum (Fig. 4B) and by the observance that its

fluorescence was quenched at alkaline pH and by metal chelators as shown in Table III. Malshet et al. (17) showed these properties are common to conjugated Schiff base structures. In order to confirm that the ARFS detected in kidney tissue in previous work (18) also contained a Schiff base structure, it was shown to be similarly affected by acidic and basic conditions and metal chelators (Table III). In contrast, the intensity of the fluorescence of retinol was unaffected by these treatments; the small decrease in the fluorescence of this compound resulted from destruction by exposure to ultraviolet light.

No phosphorus was detected in the ARFS as observed in the main fraction of fluorescent substances isolated from testes by Trombly et al. (19). Moreover, the excitation-emission maxima 355 and 490 nm of the ARFS was similar to this fraction which was isolated by silicic acid column chromatography. The ARFS was isolated as a single spot by 2-dimensional TLC in our work; hence, it could be a component of the fraction isolated by these investigators. One might expect the fluorescent substances to contain a phosphate moiety inasmuch as in vivo lipid oxidation is generally believed to involve the polyunsaturated fatty acids of phospholipids. However, should these fluorescent substances undergo partial hydrolysis by lysosomal lipases, which is highly plausible, fluorescent residues that contain no phosphorous, as detected here, might be produced as suggested by Trombly et al. (19). Moreover, the large number and complexity of fluorescent substances detected in mammalian tissue might well be explained on the basis of partial degradation of these substances by lysosomal enzymes. Although one substance was identified particularly with aging in this study, many other fluorescent substances were detected, and some of these also could be related to the formation of the aging pigment.

Although the formation of lipofuscin substances is believed to originate with the in vivo oxidation of polyunsaturated fatty acids, the results in Tables IV and V show that the intensity of the ARFS was not related directly to the unsaturated fatty acid composition of the tissues. On the other hand, the effect of age on the accumulation of ARFS is evident by comparison of the 11- and 20-month-old animals. In a somewhat analogous situation, (18) the acceleration of aging in kidneys of rats exposed to x-irradiation gave an increase in accumulation of ARFS with no apparent change in the fatty acid composition of the tissue. Because polyunsaturated fatty acids are supplied daily in the diet, there is no reason to expect that the formation of age-related fluorescent substances should have any effect on tissue fatty acid composition. Thus, it appears the fatty acid composition of the tissues, as reflected by that of the diet, influence the formation of aging pigment only insofar as aging is influenced. Other factors,

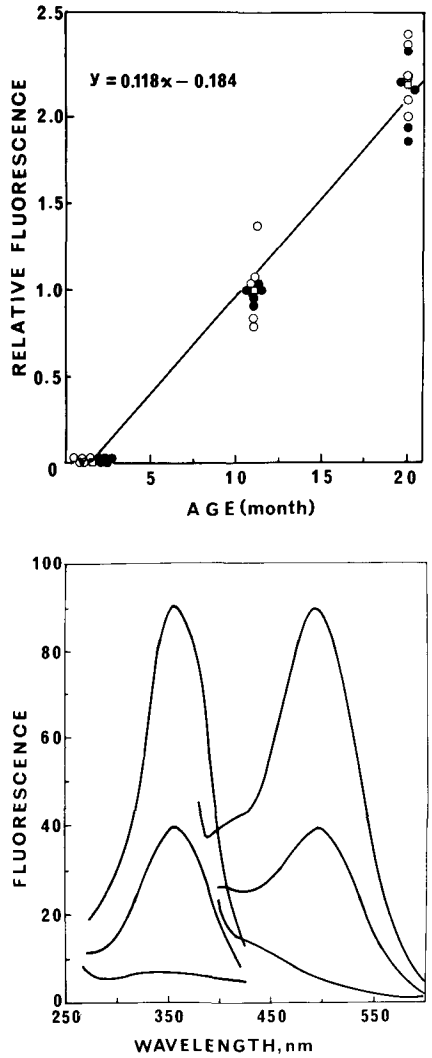

FIG. 4. Analysis of the age-related fluorescence substance (ARFS) in the testes of rats. (A) Relationship of the accumulation of ARFS with age expressed as relative fluorescence per gram of tissue, ○ = 10% corn oil diet; ● = 10% corn + menhaden oils (3:7) diet, □ = mean. Linear regression data: N = 30; correlation coefficient = 0.972; P < 0.001; slope = 0.118; Y intercept = -.184 fluorescence/age. (B) Excitation emission spectra of testicular ARFS; meter multiplier 0.001; sensitivity 50; lower curve = 8-week-old animals; middle = 11-month-old animals; upper curve = 20-month-old animals.

TABLE III

Effect of pH and Chelation on Fluorescence of ARFS

	Fluorescence maxima		Percent fluorescence		
	Ex (nm)	Em (nm)	Base[a]	Acid[b]	Chelator[c]
Testis	355	490	50.53 ± 3.61[d]	99.07 ± 1.32	83.86 ± 1.51
Kidney	355	490	48.40 ± 4.05	98.33 ± 0.35	83.51 ± 2.14
Retinol	335	478	98.83± 2.27	92.10 ± 2.23	97.44 ± 2.01

[a]Ten μ liters of 0.5N potassium methoxide in methanol added to 2 ml solution of ARFS in ethanol.

[b]ARFS ethanol solution adjusted to neutrality by addition of 10 μl of 1N acetic acid.

[c]Addition of 40 μl of 2.5 x 10^{-5} $Eu(thd)_3$ to ethanol solution of ARFS.

[d]M ± SD (n = 5).

TABLE IV

Effect of Dietary Fat and Age on the Accumulation of ARFS in Rat Testes[a]

Dietary[b] group	No. of animals	Age of animals (months)	Body wt. (g)	Testes wt. (g)	Max. fluorescent Ex (nm)	Max. fluorescent Em (nm)	Relative fluorescence per g of tissue[d]
5% SAFF	5	11	522 ± 10.3[c]	4.06 ± 0.44	355	490	0.68 ± 0.11[e]
5% HCO	4	11	351 ± 38.3	2.84 ± 0.51	355	490	0.88 ± 0.08[f]
10% Corn oil	5	11	548 ± 32.0	3.89 ± 0.18	355	490	1.01 ± 0.21[g]
10% Corn oil	5	20	513 ± 52.0	3.87 ± 0.20	355	490	2.25 ± 0.18[h]
10% Corn + menhaden	5	11	577 ± 25.8	4.04 ± 0.15	355	490	0.98 ± 0.04[i]
10% Corn + menhaden	5	20	578 ± 75.0	4.42 ± 0.47	355	490	2.11 ± 0.19[j]

[a]Fluorescence values are relative to a standard quinine sulfate solution (1 μg/ml of 0.1N H_2SO_4) with a relative fluorescence intensity of 24.

[b]See Experimental for composition of diet.

[c]M ± SD.

[d]Significances: e vs f, $P < 0.025$; g vs h, $P < 0.01$; i vs j, $P < 0.01$; f vs i, $P < 0.025$.

such as vitamin E or antioxidant levels, also might be important in this respect (20). Since the ARFS was higher in the testes of EFA-deficient animals than the normal controls, biological aging may be increased in these animals inasmuch as their life span is shorter than normal.

ACKNOWLEDGMENTS

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TABLE V

Fatty Acid Composition of Rat Testes and Serum[a]

Fatty acid	Testes				Serum	
	5% SAFF[b]	5% HCO	10% Corn oil	10% Corn - Menhaden oils (3:7)	5% SAFF	5% HCO
16:0	33.19 ± 0.67[c]	33.64 ± 0.53	33.14 ± 2.64	34.59 ± 0.70	20.85 ± 1.20	22.99 ± 0.91
16:1	1.01 ± 0.33	1.98 ± 0.18	0.97 ± 0.48	0.88 ± 0.09	1.82 ± 0.84	9.43 ± 1.20
18:0	7.52 ± 0.34	7.53 ± 0.47	6.93 ± 0.27	7.12 ± 0.07	9.78 ± 1.21	8.79 ± 1.42
18:1	11.38 ± 0.51	20.00 ± 0.04	11.98 ± 2.24	12.09 ± 0.15	9.66 ± 2.64	41.84 ± 4.69
18:2	5.18 ± 0.49	0.64 ± 0.21	7.86 ± 3.80	5.98 ± 0.80	22.64 ± 2.31	0.27 ± 0.19
20:3ω9		9.57 ± 0.07				14.21 ± 2.59
20:3ω3	0.92 ± 0.13		1.24 ± 0.25	1.68 ± 0.05		
20:4	16.28 ± 0.28	6.69 ± 0.18	14.95 ± 1.30	14.00 ± 0.08	35.25 ± 4.74	2.46 ± 1.28
22:4ω6	2.78 ± 0.12	Tr.	2.33 ± 0.41	1.77 ± 0.18		
22:5ω6	21.73 ± 0.91	12.79 ± 0.24	19.89 ± 1.83	19.30 ± 0.10		
22:6	Tr.	Tr.	0.71 ± 0.05	2.60 ± 0.26		

[a]11-month-old rats.

[b]SAFF = safflower oil; HCO = hydrogenated coconut oil, see Experimental for composition of diets.

[c]M ± SD (4 animals).

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Erucic Acid-Induced Alteration of Cardiac Triglyceride Hydrolysis

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ABSTRACT

Male Wistar rats were fed for 3 or 10 days with high erucic acid rapeseed oil (HEAR) or trierucate (TE). These diets produced increased myocardial triglyceride (TG) levels. Cardiac lipid accumulation was related to basal- and hormone- (glucagon, norepinephrine) stimulated lipolysis, determined as glycerol release, which proved to be enhanced in isolated, perfused hearts from HEAR- and TE-fed rats. Endogenous TG levels in isolated hearts from rats fed the stock and the sunflowerseed oil (SSO) diet were low and probably rate-limiting for tissue lipolytic activities. HEAR feeding of rats did not modify the rate of erucic acid (22:1) oxidation in heart. Prolonged HEAR and TE feeding led to a decrease in the endogenous TG level, a process in which the increased rate of TG hydrolysis might play an important role. The enhanced breakdown of tissue TG in hearts from TE- and HEAR-fed rats was accompanied by an increased release of fatty acids into the coronary effluent. Erucic acid was a major constituent of the perfusate fatty acids. Evidence is presented that the site of the intracellular TG breakdown is associated with lysosomes, since a subcellular fraction enriched in acid lipase, N-acetyl-β-glucosaminidase and TG could be isolated from heart homogenates of TE-fed rats. Fatty acids seemed to be an important regulator of tissue lipase activity: palmitate inhibited glucagon-stimulated lipolysis, which suggests the tissue lipase is subject to product inhibition by fatty acids.

INTRODUCTION

The effects of dietary erucic acid (*cis*-22:1 ω9) upon cardiac lipid metabolism are biphasic. During the first three days of feeding erucic acid-enriched diets, an acute myocardial triglyceride (TG) accumulation occurs (1,2). The intracellular lipidosis can be ascribed first to increased erucic acid delivery to the heart since 22:1 levels in serum very low density lipoprotein TG (VLDL-TG) (3) and myocardial lipoprotein lipase (LPL) activity are enhanced (4,5). Second, the increased intracellular levels of 22:1 are oxidized at a low rate (6,7) and 22:1 inhibits the β-oxidation of other fatty acids (8-12). This ultimately leads to increased esterification of fatty acids, mainly in TG. The fatty acid composition of the accumulated TG has been extensively documented by Kramer et al. (13) and reveals increased amounts of 22:1, 18:1, 20:1, 18:2 and 16:2, which together form the greatest fraction of TG-fatty acids.

During prolonged feeding of 22:1-enriched diets, intracellular cardiac TG levels decrease and may almost reach a normal value (14). In this process, a role of increased chain shortening of 22:1 by a peroxisomal β-oxidation system in liver and heart has been considered (15-17). Recent studies from our laboratory (17-18) have shown dietary erucic acid-induced cardiac lipidosis alters the rate of basal- and hormone-stimulated lipolysis.

In accordance with previous findings of Christiansen and Jensen (19) in heart, and of Angel (20) in adipose tissue, we have demonstrated at least part of the intracellular TG in hearts from trierucate-fed (TE) rats is membrane-limited (18). Furthermore, we presented evidence that these lipid-filled particles probably represent (secondary) lysosomes or autophagosomes. These observations seem to agree with the apparent engulfment of a lipid droplet by membrane-bound organelles observed in guinea- pig heart lysosomal fractions (21), and with the lipid-filled lysosomes observed in lipid-laden rabbit aortic cells (22).

The intracellular locus/loci of endogenous lipolytic enzymes has not been clearly shown. Evidence for a lysosomal or autophagosomal origin of endogenous lipase activity has been presented for liver (23) and for heart (18,24-25). Recently, Wang et al. (26) were able to isolate from heart homogenates a fraction enriched in TG and acid lipase.

Hormonal activation of tissue lipolysis may be mediated by phosphorylation of tissue lipase in heart, but the main regulator of tissue lipolysis in heart is possibly the intracellular fatty acid level by end-product inhibition (27-29). We have presented recent evidence that lipolysis in isolated hearts from TE-fed rats is stimulated when fatty acid oxidation is stimulated by dinitrophenol, whereas 5-gluconolactone-inhibition of fatty acid re-esterification led to a decrease in basal and hormone-stimulated lipolytic activity (28,29). These observations may indicate the activity of the tissue lipase in heart is determined by the rate of removal of product fatty acids.

The experiments presented in this paper will deal with the increased rate of lipolysis in

isolated, perfused hearts of high erucic acid rapeseed oil (HEAR)- and TE-fed rats and its relation to the increased endogenous cardiac TG pools. The role of stimulated lipolysis in the decreased TG formation and TG breakdown will be discussed. Furthermore, attention is given to the intracellular site of lipolysis, and some regulatory aspects of the enzyme are reviewed.

MATERIALS AND METHODS

Animals and Diets

Male Wistar rats (200-250 g body weight) were purchased from TNO (Rijswijk, The Netherlands). The animals had free access to food and water and were given the diets listed in Table I, supplemented with the indicated amounts of minerals and vitamins. Total fat content of the TE diet was 50%, whereas the HEAR diet contained 40% fat. The fatty acid composition of the sunflower seed oil (SSO), the HEAR and TE diets are given in Table II. The stock diet was from Hope Farms (Woerden, The Netherlands) and contained 11 energy-percent fat, 23 energy-percent protein, 66 energy-percent carbohydrate and a mineral and vitamin supplement. Before changing to another diet, the rats were fasted overnight. For the perfusion experiments, the rats were anesthetized with intraperitoneally-injected sodium pentobarbital (70 mg/kg body weight^{-1}). In all other cases, the rats were decapitated.

TABLE I

Composition of the SSO, HEAR and TE diets

Ingredients	Digestible energy (%)			g/1000 kcal		
	SSO	HEAR	TE	SSO	HEAR	TE
Casein (86% protein)	23	23	23	62	62	62
Corn starch	37	37	27	77.1	77.1	56.3
Saw dust	---	---	---	20	20	20
Sunflowerseed oil	40	---	16	43	---	17
Rapeseed oil	---	40	---	---	43	---
Trierucate[a]	---	---	34	---	---	53.8
Minerals[b]	---	---	---	5.4	5.4	5.4
Vitamins[c]	---	---	---	1.0	1.0	1.0

[a]The 22:1 content of the trierucate was 90-92%.

[b]g/1000 kcal:KCl 0.350, $MgHPO_4 \cdot 3H_2O$ 0.956, KH_2PO_4 0.475, $KHCO_3$ 0.719, $C_6H_5Na_3O_7 \cdot 2H_2O$ 0.711, $CaCO_3$ 2.014, $MnSO_4 \cdot 4H_2O$ 0.0678, $C_6H_5FeO_7 \cdot 5H_2O$ 0.044, $Cu_2C_6H_6O_8$ 0.0047, $Zn_3(C_6H_5O_7)_2$ 0.0125, KIO_3 0.00007.

[c]g/1000 kcal:choline 0.250, vitamin E 0.020, calcium silicate 0.05, myoinositol 0.025, vitamin B_{12} 0.000005, vitamin A 0.007, niacin 0.005, pantothenic acid 0.005, riboflavin 0.0015, thiamine 0.0015, vitamin D_3 0.003125, vitamin K 0.000227, vitamin B_6 0.00005, folic acid 0.00025, biotin 0.00005, filled up to 1.00 with saccharose.

TABLE II

Main Fatty Acid Composition (wt%) of Stock Diet Fats, Sunflowerseed Oil (SSO), High Erucic Acid Rapeseed Oil (HEAR) and Trierucate-sunflower Oil Mixture (TE)

Fatty acid	Stock-diet fats	SSO	HEAR	TE
16:0	13	6	3	2
16:1	3	---	---	---
18:0	3	4	---	2
18:1	33	25	16	6
18:2 [a]	42	62	13	26
18:3 + 20:0	6	---	9	2
20:1	---	---	10	2
22:1	---	---	44	60

[a]*Cis-Cis*-linoleic acid.

Perfusion Procedure

The hearts were quickly excised, chilled in precooled perfusion buffer and arranged for retrograde coronary perfusion at a rate of 300 beats/min^{-1} as described previously (30-31). The basic substrate-free perfusion buffer contained 128 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4 and 1.0 mM $MgCl_2$ and was continuously gassed with 95% O_2 and 5% CO_2 (pH 7.4, 37 C). Glucagon (2.10^{-7} M) was dissolved in perfusion medium. Norepinephrine was dissolved in buffer and infused above the aortic cannula in a final concentration of 2.10^{-7} M. When perfusion took place in the presence of [9,10-^{3}H] oleic acid, [9,10-^{3}H] erucic acid or palmitic acid, the fatty acids were neutralized with equimolar amounts of NaOH and complexed to fatty-acid-poor bovine serum albumin (BSA). BSA was defatted according to Chen (32). Before perfusion, these solutions were passed through a washed 1.2 μ Millipore membrane filter (Sartorius, Göttingen, Germany) and foaming of the albumin-containing solutions was prevented by a silicone defoamer (Antifoam A).

Hearts from TE- and HEAR-fed rats could maintain proper mechanical activity, determined as contraction amplitude or left ventricular pressure development (30), for many hours during substrate-free perfusion. Hearts isolated from rats fed with the stock or SSO diet revealed a slow and gradual loss of contractility after about 45 min of substrate-free perfusion. Control coronary flow rates (ml/min^{-1}/g wet wt^{-1}) determined by timed collection, after 15 min of preperfusion were 6.3 ± 0.1 (n = 10) for hearts from rats fed with the stock diet, 6.2 ± 0.12 (n = 10) in hearts from SSO-fed rats, 7.3 ± 0.2 (n = 10) in hearts from HEAR-fed rats and 8.0 ± 0.1 (n = 18) in hearts from TE-fed rats. After the experiments, the hearts were cut open, dried thoroughly and weighed.

Preparation of Subcellular Fractions

The procedure for the preparation of subcellular fractions from hearts of TE-fed rats is summarized in Figure 1. Two hearts were used for one fractionation. After decapitation of the rats, the hearts were removed quickly and placed in ice-cold saline (0.15 M NaCl). Major blood vessels were removed and the organs were minced with a pair of scissors. Homogenization (20% w/v in saline) was done with a Teflon pestle tissue grinder (Potter-Elvehjem) and was achieved by five up-and-down strokes with a loose fitting pestle, and the same number with a tight fitting pestle, while the pestle was rotating

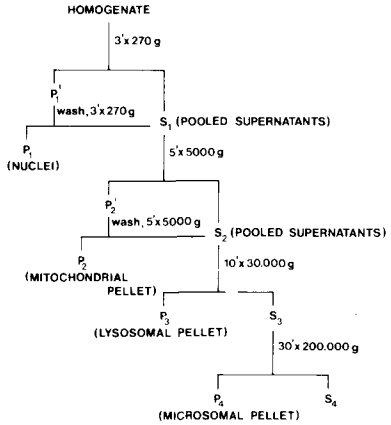

FIG. 1. Flow sheet of the isolation of mitochondrial, lysosomal and microsomal fractions from a homogenate of hearts from trierucate-fed rats. Time of centrifugation is given without acceleration.

slowly. All operations were carried out at 4 C. The nuclei and floating lipid material from the first centrifugation were discarded and the pellets obtained in the fractionation scheme were suspended in distilled water. All fractions were stored at -20 C.

Radioactive Isotope Experiments

Oxidation of oleate and erucate by isolated hearts from SSO- and HEAR-fed rats was determined during a recirculating perfusion of the hearts with 50 ml buffer containing 0.5 mM [^{3}H] erucic acid (sp act 2.2 MBq/μmol^{-1}) and 0.5 mM [^{3}H] oleic acid (sp act 3.4 MBq/μmol^{-1}) in a molar ratio to fatty acid-free BSA of 1:1. At regular intervals, perfusate samples were taken, extracted according to Bligh and Dyer (33) and 1 ml of the water phase, containing the oxidation product, ^3H_2O, was added to 10 ml of Instagel. Radioactivity was determined by liquid scintillation counting.

Assay of Enzymatic Activities

Acid lipase activity (used as a lysosomal marker) was determined at pH 4.0 with 4-methylumbelliferyl oleate as substrate according to Cortner et al. (34). A second lysosomal marker, N-acetyl-β-glucosaminidase, was also measured fluorometrically with 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranose as substrate at pH 4.4 under the same conditions as the acid lipase assay. Activity of succinate dehydrogenase, a mitochondrial marker enzyme, was determined as succinate-*p*-iodonitrotetrazolium reductase according to Meijer et al. (35). Esterase, a microsomal marker, was assayed according to Hulsmans

(36), with eserine (60 μM) present to inhibit acetylcholine esterase activity (37).

Gas Chromatographic Analysis

During control perfusion with buffer containing 1 mg fatty-acid-free BSA/ml^{-1}, and during perfusion with additional glucagon (2.10^{-7} M), total coronary effluent was collected. Effluent fatty acids were extracted according to Bligh and Dyer (33) and methylated with BF_3 (14% in methanol) as described by Morrison and Smith (38). Analysis of the methyl esters of the fatty acids was performed with a gas chromatograph (type F&M 402, Hewlett Packard) as described by Ruitenbeek (39). Retention times were compared with standard esters and mixtures of known composition. Corrections were made for residual fatty acids in the BSA-containing perfusion buffer treated with antifoam.

Analytical Methods

Glycerol in effluent samples was determined by the method of Laurell and Tibbling (40). Careful calibration of fluorescence was carried out by adding a known amount of NADH to the cuvette. Total lipids were extracted from the hearts as described by Folch et al. (41) and TG determined according to Laurell (42). Protein was estimated by the procedure of Lowry et al. (43) and effluent fatty acids according to Duncombe (44).

Reagents

Reagents (all of analytical grade) were obtained from Merck (Darmstadt, Germany), bovine serum albumin (fraction V) and borontrifluoride (BF_3) from Sigma (St. Louis, MO). Sodium pentobarbital was obtained from Abbott (Saint-Remy sur Avre, France) and Antifoam A from Dow Corning (Midland, MI). The enzymes and cofactors for the determination of glycerol were from Boehringer and Sons (Mannheim, Germany). [^{3}H]Oleic acid was purchased from the Radiochemical Centre (Amersham, England), and Instagel from Packard Instrument Company (Downers Grove, IL). [^{3}H]Erucic acid was synthesized by Unilever Research (Vlaardingen, The Netherlands).

Statistical Analysis

Most results are given as mean ± standard error of the mean (SEM); n is the number of experiments. Values of P were calculated with Student t-test (two-tailed). $P > 0.05$ was considered not significant (NS).

RESULTS AND DISCUSSION

Myocardial Lipidosis, Lipolysis and Erucate Oxidation

Dietary TE and HEAR lead to a tremendous rise in endogenous cardiac TG levels: 33.6 ± 2.6 μmol TG/g myocardial protein^{-1} in hearts (n = 8) from rats fed with the stock diet, 300 ± 33 in hearts (n = 3) after a 3 day diet with HEAR and 716 ± 33 in hearts (n = 7) after a 3 day diet with TE. The 22:1-induced TG accumulation is well known and illustrates a marked alteration in the TG metabolsim of the heart. Since fatty acid turnover in heart is high, a minor imbalance between fatty acid supply and oxidation rate will rapidly promote fatty acid esterification and intracellular accumulation of TG (45), at least partly, in membrane-limited particles which may represent lipid-filled (secondary) lysosomes or autophagosomes (17-19,21,26).

The activity of endogenous TG hydrolyzing enzyme(s) in isolated, perfused hearts can be determined from the rate of glycerol released in the coronary effluent. The effect of HEAR and TE feeding of rats on basal- and hormone-(norepinephrine) stimulated glycerol release from the hearts during substrate-free perfusion is presented in Figure 2. The low basal rate of lipolysis observed in hearts from rats fed with the stock or SSO diet is markedly increased after feeding a high fat diet rich in 22:1 (HEAR and TE) while no difference could be observed between the rates of lipolysis in hearts from

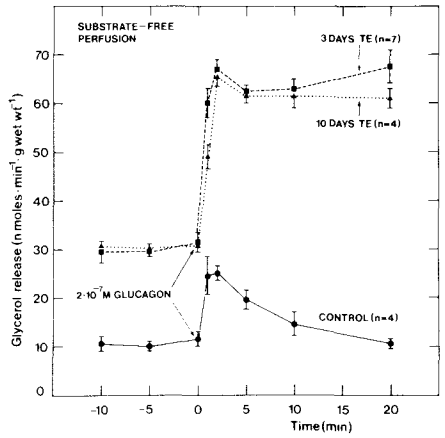

FIG. 2. The effect of dietary sunflowerseed oil (SSO), high erucic acid rapeseed oil (HEAR) and trierucate (TE) upon basal and norepinephrine-stimulated glycerol release from isolated, substrate-free perfused rat hearts. Control values were obtained from hearts of rats fed with the stock diet. The data are the mean ± SEM of the indicated number (n) of experiments.

HEAR- and TE-fed rats. Basal and norepinephrine-stimulated lipolysis in hearts, obtained from rats after a 3-day diet with SSO, did not differ significantly from lipolysis in hearts from rats fed with the stock diet while no TG accumulation occurred: 33.6 ± 2.6 μmol TG/g myocardial protein^{-1} in hearts (n = 8) from stock-fed rats and 27.7 ± 2.9 in hearts (n = 4) from SSO-fed rats, NS. Norepinephrine-stimulated glycerol release is of short duration in hearts from stock-fed rats, but prolonged in TG-enriched hearts. The low basal rates and the transient hormonal stimulation of lipolysis argue for limited availability of endogenous TG in isolated, perfused hearts from rats fed with the stock diet (46). The correlation between endogenous TG availability and the rate of tissue lipolysis is also present in hearts from fasted and diabetic rats (47) where intracellular TG accumulation is accompanied by an increased rate of glycerol release during perfusion. In contrast to our experiments, Hsu and Kummerow (48) could not demonstrate hormonal stimulation of lipolytic activity in extracts of hearts from HEAR-fed rats.

In addition to increased tissue TG levels, other factors may be involved in the modification of tissue lipase activity in lipid-enriched hearts from HEAR and TE-fed rats. For instance, increased serum corticosterone and testosterone levels as a consequence of feeding rats diets rich in very long chain fatty acids, as reported by Hülsmann (49), may be a complementary regulating factor, especially since we have found glucocorticoids exert a permissive action on lipolysis in hearts from HEAR-fed rats (18).

It has been known for some time (1-3,14-16) that upon prolonged feeding of a high fat diet rich in 22:1, rat cardiac TG levels reach a maximum after about 4 days and then tend to decrease gradually. We found that after 10 days of TE feeding, myocardial TG content had declined from 716 ± 33 (n = 7) to 509 ± 57 (n = 4) μmole TG/g myocardial protein^{-1} ($P < 0.05$). The decrease in TG levels did not affect endogenous lipolysis, since basal- and glucagon-stimulated glycerol release in hearts from rats fed 3 and 10 days with TE were not statistically different (Fig. 3).

Several mechanisms may be involved in the well known limitation of TG accumulation in heart. The decreased presence of 22:1 in VLDL-TG (3) and increased chain-shortening of 22:1 in the myocardium itself (16) may lead to a decreased intracellular 22:1 level. These actions result in decreased fatty acid esterification and TG formation. The question remains by which mechanism the TG content will

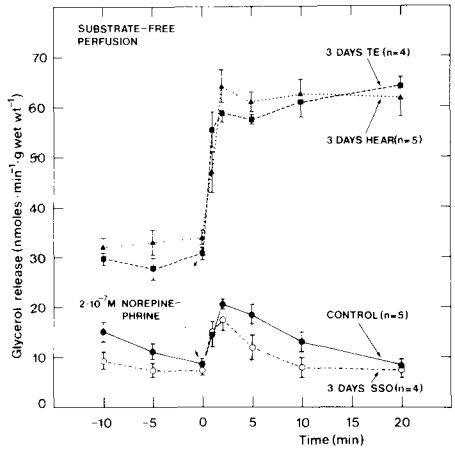

FIG. 3. The effect of prolonged trierucate (TE) feeding upon basal and glucagon-stimulated glycerol release from isolated, substrate-free, perfused rat hearts. See legend to Fig. 2.

decline. It has been demonstrated (10) that the rate of oxidation of 22:1 in heart is slower compared to the oxidation rate of 16:0. To investigate whether 22:1 feeding would enhance the capacity of the heart to oxidize 22:1 (in other words, whether the "oxidative machinery" could adapt toward a specific fatty acid), hearts from HEAR- and SSO-fed rats were perfused with [^{3}H]erucic acid and the rate of 22:1 oxidation was compared with [^{3}H]oleic acid oxidation in both groups of hearts. It proved hearts from HEAR and SSO-fed rats oxidized 22:1 at the same rate (2.3 vs 2.1 μmol 22.1/hr^{-1}/heart^{-1}, (n = 2) while the oleate oxidation rate was higher but again not different in both groups of hearts (4.8 μmol 18:1/hr^{-1}/heart^{-1}, n = 2). From these data, we conclude that 22:1 feeding does not enhance 22:1 oxidation in heart. Our data agree with previous findings in mitochondrial preparations isolated from hearts of rats fed rapeseed oil (50). Therefore, the events leading to a decline in TG content are: (a) increased chain shortening of 22:1 by a peroxisomal, cyanide insensitive, β-oxidation system in the liver, resulting in a decreased 22:1 content in the blood lipids and concomitantly to a reduced 22:1 delivery to the heart (3,17,51), (b) increased capacity for chain shortening of 22:1 in hearts from HEAR- and TE-fed rats (16) and (c) increased TG hydrolysis.

One consequence of increased TG breakdown in hearts of TE-fed rats could be stimulated fatty acid release from the heart. In Tables III and IV, total release of fatty acids and their relative composition are presented.

TABLE III

Glycerol and Fatty Acid (FA) Release from Isolated Hearts of Trierucate-fed Rats[a]: Effect of Glucagon

Condition	Glycerol release	Fatty acid release	FA/glycerol ratio
	nmoles/min^{-1}g wet wt^{-1}		
Control	30.2 ± 1.4[b]	17.3 ± 4.7[b]	0.53 ± 0.12
Control + 2.10^{-7} M glucagon	62.1 ± 1.2[c]	19.1 ± 2.5[c]	0.31 ± 0.04

[a]The hearts were obtained from rats fed for 3 days with the TE diet.

[b]Mean glycerol or fatty acid release ± SEM during the 15 min preperfusion (control), n = 4.

[c]Mean glycerol or fatty acid release during a subsequent 15 min perfusion in the presence of 2.10^{-7} M glucagon, n = 4.

TABLE IV

Relative Composition of Fatty Acids in Effluent from Perfused Hearts of Trierucate-fed Rats: Effect of Glucagon

Fatty acid (wt %)[a]	Control		+ 2.10^{-7} M glucagon
12:0	3.4 ± 1.1[b]		2.9 ± 0.4
14:0	4.4 ± 0.4		4.3 ± 0.5
14:1	1.7 ± 0.5		2.2 ± 0.5
16:0	23.8 ± 1.7	$P < 0.01$	16.6 ± 1.3
16:1	7.8 ± 1.5		4.0 ± 0.4
18:0	12.8 ± 0.8	$P < 0.05$	17.6 ± 0.9
18:1	16.8 ± 0.9	$P < 0.01$	13.0 ± 0.9
18:2	4.3 ± 1.0		6.5 ± 2.0
18:3 + 20:0	2.3 ± 0.3		3.0 ± 1.1
20:1	2.2 ± 0.5		3.4 ± 0.4
20:3	1.7 ± 0.2		3.8 ± 0.5
20:4 + 22:0	2.2 ± 0.3		2.9 ± 0.6
22:1	16.1 ± 2.1		19.3 ± 1.3

[a]Indicated number of carbon atoms: number of double bonds.

[b]All data are the mean ± SEM of 4 separate experiments; when no P value is indicated the data were not significantly different.

Assuming that endogenous triglyceride hydrolysis is complete (TG → glycerol + 3 fatty acids), it can be calculated that during control perfusion about 20% of the liberated TG-fatty acids are recovered in the perfusate, whereas in the presence of glucagon only 10% is released from the hearts. This may be explained by more fatty acid oxidation in the presence of glucagon when the mechanical activity of the hearts is increased and by more fatty acid re-esterification when glycerol-3-phosphate becomes available by glucagon-stimulated glycogenolysis (5). Fatty acid release from substrate-free perfused hearts from TE-fed rats is about 10 times higher than fatty acid release from hearts of rats fed with the stock diet perfused in the presence of 11.1 mM glucose which amounted 1.82 (1.77 and 1.88) nmoles/min^{-1}/g wet wt^{-1}. The fatty acids mainly released from the TG-enriched hearts are 16:0, 18:0, 18:1 and 22:1 (Table IV). The significant decrease in percent 16:0 and 18:1 released during perfusion in the presence of glucagon probably results from preferential oxidation of these substrates for energy metabolism when contractility is increased by glucagon. The significant rise in percent 18:0 released may suggest sluggish use of this fatty acid. The increased fatty acid release may be involved in the enhanced coronary flow rates observed in hearts from HEAR- and TE-fed rats (see Materials and Methods). Earlier, coronary vasodilatory properties of medium and long chain fatty acids were demonstrated (52).

Subcellular Origin of Endogenous Lipolytic Activity

Two lipases are presumably present in heart (19). First, a lipoprotein lipase, which interacts

with chylomicrons and VLDL may be localized on the endothelial lining of the coronaries. This enzyme can be removed almost completely from the heart by heparin perfusion. Second, there is a tissue lipase, which is the intracellular locus of lipolysis (17-18,21,26). In heart, the tissue lipase is probably of lysosomal origin since chloroquine, a lysosomal inhibitor, could inhibit TG breakdown (17). Furthermore, a chloroquine-inhibition of adipose tissue lipolysis was reported (17) and a chloroquine-sensitive ketone body formation in liver was demonstrated (53-54). These findings suggest that also in these tissues, a lysosomal lipase is involved in the hydrolysis of endogenous TG. Moreover, the demonstration of lipid-filled lysosomes in heart (5,18) is an additional indication that lysosomes are important in myocardial TG turnover. To investigate the subcellular distribution of marker enzymes and triglycerides, hearts from TE-fed rats were homogenized in 0.15 M NaCl and subcellular fractions obtained as illustrated in Figure 1. The use of ionic extraction solutions in preparative methods, giving a better yield and quality of lysosomal membranes, has been advocated recently by Ruth et al. (55). The subcellular distribution of markers and TG is presented in Figure 4. The lysosomal fraction (**L**) appeared to be enriched in two lysosomal marker enzyme activities (acid lipase and N-acetyl-β-glucosaminidase) and TG, whereas the contamination with mitochondrial membranes, estimated from the succinate dehydrogenase activity, is low. Although the **L**-fraction is contaminated with microsomal membranes, it still contains appreciable esterase activity, and the distribution pattern of the TG-containing fractions suggests lysosomal instead of microsomal enrichment with TG. Therefore, lysosomes in hearts from TE-fed rats may be sites of the lipase (acid) activity, since lysosomes are enriched both in acid lipase and TG (5,29).

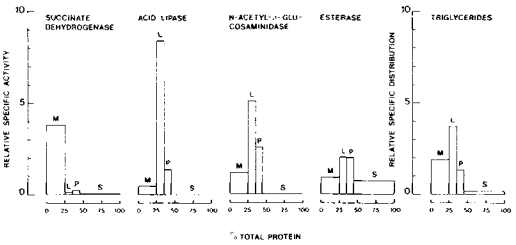

FIG. 4. Distribution patterns of enzymes and triglycerides in rat heart subcellular fractions separated as presented in Fig. 1. From left to right in their order of isolation: heavy mitochondrial (**M**), lysosomal (**L**), microsomal (**P**), and final supernatant (**S**) fractions. Relative specific activity is defined as % of total activity/% of total protein^{-1}. Relative specific distribution is defined as % of total triglycerides/% of total protein^{-1}. Enzyme activities are enriched in the subfractions if the relative specific activity is greater than one.

Regulation of Triglyceride Hydrolysis

In agreement with a previous report (48), we have found the lipase activity in homogenates of hearts from TE-fed rats, determined with added trioleate as substrate, is low, whereas with umbelliferyl-oleate as substrate, lipase activity is higher. On the other hand, during incubation of a 500 g supernatant of a homogenate from a TG-enriched heart at pH 7.0 and 37 C, continuous glycerol production can be estimated (14.3 nmoles/min^{-1}/heart^{-1}). This rate of glycerol production is lower still in comparison with the rate of lipolysis in the isolated, substrate-free perfused heart of a TE-fed rat (30-34 nmoles/min^{-1}/heart^{-1}). The depressed lipase activity in these heart homogenates or subcellular fractions probably results from product inhibition of the tissue lipase by the liberated fatty acids. Crass III et al. (27) demonstrated that TG breakdown in the isolated, perfused heart was suppressed when fatty acids were present during perfusion, and Hron et al. (56) showed inhibition of glycerol release by octanoate (8:0). In Figure 5, 0.25 mM palmitate, complexed to fatty-acid-free BSA in a molar ratio of 4:1, depressed the glucagon-stimulated glycerol release in hearts

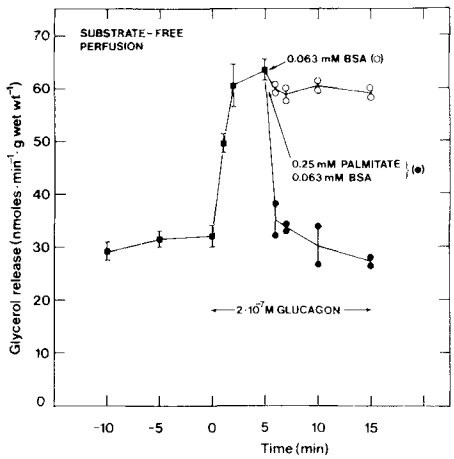

FIG. 5. The effect of 0.25 mM palmitate and 0.063 mM bovine serum albumin (BSA) upon glucagon-stimulated glycerol release from isolated, substrate-free, perfused hearts of rats fed for 3 days with TE. The palmitate was complexed to fatty acid-free BSA in a molar ratio of 4:1. The first six points ($t = -10 \rightarrow t = 5$) are the mean ± SEM of 4 perfusions. The data of two subsequent, separate perfusions, illustrating the palmitate and BSA effect, are presented.

from TE-fed rats. This high fatty acid: BSA molar ratio was used to assure a high palmitate uptake by the heart (see 57 for a review). Since fatty acids do not affect myocardial adenyl cyclase activity (49), the inhibitory action of 16:0 is probably directly caused by feedback regulation of the tissue lipase activity. In previous reports (28,29) we showed mechanisms leading to enhanced fatty acid oxidation (increased contractility, uncoupling of oxidative phosphorylation) lead to stimulated glycerol release (lipase activity) in hearts from TE-fed rats. Thus, besides a possible direct hormonal regulation of the tissue lipase activity, for which no clear-cut evidence has been presented, inhibition by end-product fatty acids is an important regulating mechanism in cardiac TG hydrolysis (54).

In summary, our experiments indicate the TG pool in isolated, perfused hearts from stock diet-fed rats is small and probably rate-limiting for endogenous lipolysis. The increased cardiac TG content by dietary 22:1 is accompanied by increased basal- and hormone-stimulated lipolysis. We propose this enhanced rate of endogenous TG hydrolysis is responsible partly for the decreased tissue TG levels observed during prolonged 22:1 feeding. Increased lipolysis is associated with fatty acids released from the perfused heart, with 22:1 as a main constituent of the liberated fatty acids. TG storage and hydrolyzing enzyme(s) are probably of lysosomal origin and product fatty acids may be predominant in the regulation of the tissue lipase activity.

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Essential Fatty Acid Restriction Inhibits Vitamin D-Dependent Calcium Absorption

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ABSTRACT

Essential fatty acid (EFA) restriction has been found to inhibit the action of vitamin D on the active transport of calcium in the intestine. This inhibition suggests EFAs are involved in facilitating the active transport of calcium across the mucosal membrane.

INTRODUCTION

It is recognized that 1α,25-dihydroxycholecalciferol (1α,25$(OH)_2D_3$) is the active hormonal form of vitamin D_3 and that the intestine is one of its major target organs. The focus is now on the hormone's mode of action in the gut; one major physiological effect is a stimulation of intestinal calcium transport (1-3). The biochemical basis of this action is, however, still not clear.

In the chick, one change ascribed to this hormone is an alteration in the brush-border lipid composition of intestinal mucosal cells (4,5). Goodman et al. (4) reported vitamin D treatment led to an alteration in the microvillar total phosphoglyceride fatty acid composition of vitamin D-deficient chicks. Rasmussen et al. (5) observed that 1α(OH) D_3 administered to vitamin D-deficient chicks resulted in an increased total lipid phosphorus of duodenal brush-border membranes; they also noted a reorganization of the choline phosphoglyceride (CPG) fatty acids with an increase in linoleic (18:2 n-6) and arachidonic (20:4 n-6) acids. These lipid changes in the vitamin D-supplemented animals were associated with increased calcium transport.

In the rat, we have also observed changes in the intestinal fatty acid composition of vitamin D-deficient animals following treatment with 1α,25$(OH)_2D_3$ (6). The fatty acid changes were more pronounced in the CPG fraction of the smooth muscle where there was decreased linoleic acid and increased arachidonic acid. On the other hand, in the mucosal CPG fraction, there was increased linoleic acid. These lipid changes in the 1α,25$(OH)_2D_3$-dosed rats were also associated with increased calcium transport (6).

More recently, O'Doherty (7) has reported (a) 1α,25$(OH)_2D_3$ stimulates the activity of the intestinal phosphatidylcholine (PC) deacylation-reacylation cycle, and (b) this stimulation in the activity of the enzymes may be the mechanism for the reported fatty acid compositional changes of the choline phosphoglyceride fractions in the intestinal cell membranes. Such fatty acid changes could modify the fluidity and permeability properties of the membranes, thus facilitating calcium transport.

Linoleic and arachidonic acids are important constituents of the structural lipids. Both acids are involved in membrane permeability (8), membrane-bound enzyme and ion transport (9) and both are precursors of prostaglandins (10,11). The possibility exists, therefore, that an increased intestinal calcium transport could be associated with one, or indeed all, of the above parameters.

To investigate these possibilities further, we examined the influence of dietary linoleic acid and of an inhibitor of prostaglandin synthesis on the calcium transport in vitamin D-deficient and vitamin D-treated rats.

MATERIALS AND METHODS

Weanling inbred Wistar albino strain rats were raised for 3 wk on a semisynthetic vitamin D-deficient diet. The composition of the diet is presented in Table I. Arachis oil provided the linoleic acid. After 1 wk on this diet, 18 rats were provided an essential fatty acid (EFA)-deficient diet by replacing arachis oil with hydrogenated coconut oil. The remaining 32 rats were maintained on the arachis oil diet. After an additional 2 wk, the animals were used in the experiments. However, 3 days before killing, half the animals on both diets were given vitamin D (orally, 100 iu, 2.5 μg/rat). Also, half the rats on the arachis diet, including vitamin D-deficient and D-replete rats, received 2 doses of indomethacin (intraperitoneally) at 0.2 mg/kg. body wt. at 28 hr and 4 hr before

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killing.

The rats were killed by cerebral fracture and the intestine removed immediately for the gut-sac preparation. Details of the incubation procedure are given by Hill et al. (1).

RESULTS

The in vitro calcium transport in the gut-sac preparations from the different dietary groups are presented in Table II, expressed as the ratio of ^{45}Ca serosal:mucosal. This ratio was low at 1.8 in the vitamin D-deficient animals either on the EFA-adequate or EFA-deficient diets. The group injected with indomethacin had a similar low ratio. However, the animals that were supplied with vitamin D 3 days before the experiment and were also on an EFA-adequate diet showed an increased calcium transport with a ratio of 3.4. No such increase in the calcium transport ratio was observed in the group repleted with vitamin D but fed an EFA-deficient diet; calcium transport in the vitamin D-supplemented EFA-deficient animals was significantly lower than in the vitamin D-supplemented EFA-replete animals ($P < 0.005$). The D-replete, EFA-adequate group dosed with indomethacin showed an even higher calcium transport, however, than the D-replete, EFA-adequate group ($P < 0.05$).

Weight gain in the rats on the EFA-deficient diet was lower than in animals in the EFA-adequate diet. The weights are given in Table III for the 2 wk period the animals were on the diet.

TABLE I

Composition of the Vitamin D-Deficient Semisynthetic Diet

Diet	g
White flour	4,250
Egg albumin	500
NaCl	100
$CaCO_3$	47.5
Ferric citrate	10
Disodium hydroxy orthophosphate	112
Vitamin mix	20[a]
Arachis oil	220
Total	5,000 g

Vitamin	Percent composition
Aneurine HCl	0.5
Riboflavin	0.5
Pyridoxine HCl	0.5
Calcium pantothenate	2.8
Nicotinamide	2.0
Inositol	20.0
Folic acid	0.03
Biotin	0.01
Vitamin B_{12}	0.003
Glucose monohydrate	73.7

[a]Water soluble vitamin mixture 20 g/5 kg diet.

DISCUSSION

It has been suggested that the changes in the fatty acid composition of the intestinal cell membranes observed in vitamin D deficiency has an influence on the calcium transport (5). Such fatty acid changes, by modifying the fluidity and permeability of the membrane, may influence calcium transport. Alternatively, prostaglandin synthesis may also influence the transport mechanism.

Our results show quite clearly that EFAs do affect the vitamin D-dependent transport of calcium across the intestinal mucosa. An EFA-deficient diet fed to the rats for 2 wk was sufficient to negate the effect of vitamin D on calcium absorption, and it can be seen from the results in Table III that calcium absorption is the same in D-replete animals fed an EFA-deficient diet as it is in the D-deficient controls.

Why calcium absorption in rats is affected by an EFA-deficient diet cannot be answered by our results. The mechanism of action affected by EFAs may involve fluidity or permeability changes in the membrane. If prostaglandins facilitate calcium absorption, indomethacin dosed animals might have been expected to have had a lower calcium absorption than their respective undosed controls, whereas in our experiment the reverse is true. The D-replete, EFA-replete animals dosed with indomethacin had a higher calcium absorption than the undosed D-replete, EFA-replete controls.

We have already shown (6) that $1,25(OH)_2D_3$ will alter the fatty acid composition of phosphoglycerides in the mucosa and smooth muscle of D-deficient, EFA-replete animals and that the main changes observed are in the concentrations of linoleic and arachidonic acids—both prostaglandin precursors. The reults reported here would suggest that, if prostaglandins are involved in calcium absorption, it may be at a stage of one remove from the initial step facilitating entry of calcium to the cell. Prostaglandins perhaps limit the entry of calcium to the cell. Inhibiting prostaglandin synthesis with indomethacin may remove this limitation and permit more calcium to cross the mucosa. This suggestion could explain the increased calcium absorption which we observed in the indomethacin dosed animals.

TABLE II

The Ratio of ^{45}Ca Serosal/Mucosal in Everted Gut Sacs from Rats Maintained on Different Dietary Regimes[a]

	EFA-adequate (arachis oil)	EFA-deficient (hydrogenated coconut oil)	EFA-adequate plus indomethacin
Vitamin D-deficient	1.8 ± 0.56 (n = 7)	1.8 ± 0.22 (n = 8)	1.5 ± 0.37 (n = 8)
Vitamin D-treated	3.4 ± 0.84[b] (n = 8)	2.0 ± 0.42 (n = 8)	4.4 ± 0.68[b] (n = 7)

[a]Results expressed as Mean ± SD.

[b]Significantly different from equivalent D-deficient control (*P<0.005).

TABLE III

Weight Gain (g) in Vitamin D-deficient Rats Fed Essential Fatty Acid (EFA)-adequate and EFA-Deficient Diets[a]

	Days on diet		
EFA status	0	7	13
EFA adequate (n = 16)	55.06 ± 7.16	62.56 ± 8.21	73.81 ± 8.23
EFA adequate given indomethacin (n = 16)	55.75 ± 7.92	67.31 ± 10.17	81.87 ± 11.8[b]
EFA deficient (n = 18)	55.4 ± 7.16	59.16 ± 7.69	68.2 ± 11.72

[a]Values expressed as mean ± SD.

[b]Significantly different from EFA adequate group (P<0.05) and from EFA deficient group (P<0.01).

Effects of vitamin D and its metabolites on the composition of phosphoglycerides have been reported by Goodman et al. (4), Rasmussen et al. (5) and ourselves (6). Recently, O'Doherty (7) reported that $1\alpha,25(OH)_2D_3$ stimulates the activity of the intestinal phosphatidyl deacylation-reacylation cycle. He suggested this stimulation might be the mechanism for the changes in fatty acid composition reported by the other groups. Our results take these observations one stage further and show that the reverse is also true and that a deficiency of EFAs will affect vitamin D-dependent calcium absorption in the gut. Our results were obtained with animals dosed with vitamin D 72 hr beforehand. It is still unknown whether the same effect can be obtained with the active metabolite $1\alpha,25(OH)_2D_3$ in the time interval during which this metabolite is known to increase calcium absorption in the gut. If it is, EFAs will be shown to have a direct role in the mechanism of action of calcium entry into the intestinal cell. This work is in progress.

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Mitochondrial Metabolism of (D,L)-Threo-9, 10-Dibromo Palmitic Acid

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ABSTRACT

Bromination of palmitoleic or palmitelaidic acid proceeds by *trans* addition and yields dibrominated products which cannot undergo β-oxidation when incubated with mitochondria isolated from hamster brown adipose tissue. These mitochondria were selected because they have a high capacity for oxidation of C_{16} fatty acids and because they are readily uncoupled by an excess of free fatty acids of this chain length. The only metabolites which could be recovered from the incubation mixtures were dibromopalmitoylcarnitine and dibromopalmitoyl CoA. Free fatty acid was also recovered. Addition of synthetic carnitine or CoA esters of brominated fatty acids did not interfere with subsequent oxidation of palmitoylcarnitine. Addition of the free brominated fatty acids did significantly increase the rate of oxidation of subsequent additions of palmitoylcarnitine, as did other known synthetic uncouplers. These results are consistent with observations by others that feeding brominated oils leads to brominated fatty acid incorporation into tissue lipids, and indicate why this is so. They also provide a possible explanation for the hepatic damage noted in feeding experiments.

Brominated oil feeding leads to accumulation of brominated fats and to evidence of hepatic injury in subject animals (1-3). Since the major site of fatty acid oxidation is the mitochondria, the experiments reported here were addressed to the question of how brominated fatty acids are metabolized by isolated mitochondria. It has long been known that mitochondria isolated from brown adipose tissue (BAT) have an unusually high capacity to oxidize fatty acids (4). In addition, these mitochondria, when freshly isolated, show very loose coupling of oxidation to phosphorylation. When suspensions are treated with carnitine and ATP they can readily be brought to a more tightly coupled state (5) and they then exhibit a sensitivity to uncoupling effects of excess free fatty acids several times that of liver mitochondria (4,6). For these reasons, we selected BAT mitochondria as a sensitive and effective means of studying the metabolism of 9,10-dibromo palmitic acid prepared by direct bromination of either palmitoleic or palmitelaidic acid. Using this system we have shown that brominated fatty acids cannot undergo normal β-oxidation.

MATERIALS AND METHODS

[U-^{14}C]Palmitic acid was obtained from New England Nuclear Corp. (Boston, MA) and diluted to an appropriate specific activity (sp act) with unlabeled material purchased from the Hormel Institute (Austin, MN) or from Sigma Chemical Co. (St. Louis, MO). It was dissolved in 95% ethanol to give a final concentration of 5.4 mM. The radioactivity was determined by liquid scintillation counting.

[U-^{14}C]Palmitoleic acid was obtained from Dohm Products, Ltd., N. Hollywood, CA. It was diluted as required with unlabeled material purchased from Sigma Chemical Co. Since this supplier was unable to furnish additional material, some of the later experiments were performed with a sample of [1-^{14}C]palmitoleic acid purchased from Applied Sciences, Bellefonte, PA.

(D,L)-threo-9,10-Dibromo palmitic acid (DBPA) was prepared by direct bromination of the monoenoic acid. A 10% (v/v) solution of bromine in CCl_4 was added dropwise to a vigorously stirred solution of the free fatty acid in the same solvent. Solutions were thoroughly chilled in ice baths and exposure to bright light was avoided. Bromine was added until a calculated 5% molar excess was present, the solution was stirred for an additional 15-30 min, then the brominated product was washed with 10% $NaHSO_3$, 10% NaCl and water, in that order. The organic phase was dried over anhydrous magnesium perchlorate, filtered and solvent removed at room temperature in a rotary evaporator. The product was a colorless thick oil at room temperature. When examined by proton nuclear magnetic resonance (NMR) and by mass spectrometry (MS), no evidence of α-bromination was detectable.

When prepared from [U-^{14}C]palmitoleic acid, the product was adjusted by dilution with unlabeled material to a sp act of 100 μCi/mmol. When prepared from [1-^{14}C]palmitoleic acid, the final sp act was adjusted to 23 μCi/mmol.

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(D,L)-threo-9,10-Dibromopalmitoyl chloride (DBPC1) was prepared by heating free DBPA acid for 18 hr at 45-50 C with redistilled thionyl chloride in the presence of a catalytic concentration of dried dimethylformamide using the precautions described by Bosshard et al. (7). Excess reagent and solvents were removed with a rotary evaporator at room temperature.

(D,L)-threo-9,10-Dibromopalmitoyl-L-carnitine (DBPC) was prepared as follows: L-carnitine benzyl ester, prepared according to Brendel and Bressler (8), was acylated with the bromo-acid chloride (DBPC1) prepared as we have outlined. After acylation the product was dissolved in ethanol and hydrogenated at room temperature in the presence of platinum oxide. The free acylcarnitine was precipitated with isopropyl ether, thoroughly washed with petroleum ether (30-60) and dried. The product gave a single spot on silica gel (thin layer chromatography (TLC) plates developed with chloroform/methanol/0.1 M sodium acetate (80:80:20) with an R_f = 0.6. This is comparable to the value observed for unbrominated palmitoyl carnitine. Free carnitine did not move from the origin under these conditions, and free fatty acids migrated with the solvent front. In addition, the product gave an orange-yellow precipitate with Dragendorff's reagent (Bregoff-Delwiche modification as described by Stahl) for quaternary bases (9).

(D,L)-threo-9,10-Dibromopalmitoyl CoA (DCPCoA) was synthesized by the Seubert method (10). Thioester bonds in the product were detected by measuring the change in optical density at 232 nm before and after alkaline hydrolysis. No free fatty acid could be detected in the product by TLC.

Isolation of Mitochondria

Commercially procured male golden hamsters (*Mesocricetus auratus*) with an initial body wt of 90-100 g were cold acclimated at 4 C for at least 4 wk with free access to food and water. Prior to sacrifice the animals were fasted for 24 hr. Animals were sacrificed by decapitation and exsanguination and the brown adipose tissue was collected from the interscapular, cervical and thoracic deposits. The excised tissue was quickly chilled in 0.3 M sucrose and the mitochondria isolated as previously described (11). The washed mitochondria were finally suspended in 0.3 M sucrose at a protein concentration of 35-50 mg protein/ml.

Measurement of Oxygen Uptake

Mitochondrial oxygen uptake was determined polarographically with a Clark type electrode system, using the apparatus and standardization procedures previously described (11). For each experiment, ca. 1 mg of mitochondrial protein was suspended in 1 ml of a buffer containing 50 mM KC1, 6 mM $MgC1_2$, 8 mM N-tris[hydroxymethyl]-2-amino-ethanesulfonic acid acid (TES), 1 mM ethylenediamine tetraacetate (EDTA), 23 mM sucrose, 3.3 mM K Malate and 14 mM KH_2PO_4, at pH 7.4. Coupling was induced by the addition of 2.5 mM ATP, 5 nM CoA and 3.3 μM DL-carnitine. Each mitochondrial preparation was further checked for tightness of coupling by observing the several-fold stimulation in the maximal oxidation rate of added L-palmitoylcarnitine caused by the addition of carbonyl cyanide trifluoromethyl phenylhydrazone (FCCP).

In experiments where it was desired to recover added radiolabeled materials, a 10 ml polarographic cell was employed, with all additions scaled upward, accordingly.

Recovery of Labeled Metabolites

Aliquots of mitochondria (6-7 mg), coupled as already described, were treated with a single addition of L-palmitoylcarnitine (37.5 nmol) as a routine check of metabolic state. After this addition, the respiratory rate quickly returned to basal values. Within the next minute, 100 nmol of ^{14}C-DBPA was added. One minute thereafter, a second addition of L-palmitoylcarnitine was made and again the respiratory rate was allowed to return to a basal rate.

The entire contents of the cell was transferred to a centrifuge tube. The cell was washed with 10 ml of fresh suspending buffer which was also added to the centrifuge tube. The mitochondria were then pelleted at 5 C and the supernatant carefully decanted. The pellet was resuspended in fresh buffer, centrifuged again, and the washed pellet resuspended in 1.375 M KC1. Between experiments, the cell was extensively washed with water, 95% ethanol, isolation medium, 95% ethanol, 1% bovine serum albumin and fresh isolation medium in order to remove strongly absorbed dibromo palmitic acid.

The collected pellets and supernatants from several experiments were combined and extracted according to the Bligh and Dyer procedure C (12). Following the extractions, both chloroform and methanol-water portions were dried at room temperature in a nitrogen evaporator, then redissolved in a known volume of chloroform. For column chromatographic separation, each sample was applied to a silicic acid column, 11 cm x 2 cm, prepared according to Hirsch and Ahrens (13).

The columns were eluted with 3 separate

solvent systems (14), composed as follows: Solvent I: $CHCl_3/CH_3OH/NH_4OH/H_2O$ (100:70:6:6, v/v), Solvent II: $CHCl_3/CH_3OH/CH_3COOH$(17 N)/H_2O (100:50:16:8, v/v), Solvent III: n-$CH_3(CH_2)_3OH/CH_3COOH$(17 N)/H_2O (5:2:3, v/v). In each instance, 100 fractions (1 ml) of Solvent I were collected before passage to the next solvent.

Fractions were added to Tritosol and counted in a Beckman liquid scintillation counter.

Detection of $^{14}CO_2$ Production

Aliquots of freshly prepared BAT mitochondria were precoupled by the treatment already described, with constant magnetic stirring for 10 min. At the end of the interval, when respiratory rates had subsided to basal values, aliquots of 0.73-0.75 ml were added to 50 ml Erlenmeyer flasks fitted with a plastic center suspended from a pierced, tightly fitting rubber septum (Kontes Glass Co., Vineland, NJ). The center wells were fitted with fluted strips of filter paper wet with 0.25 ml of 10% KOH. Additions of either [U-^{14}C] palmitic acid or[U-^{14}C] DBPA were made by placing the requisite amount of reagent in 6 mm x 10 mm test tubes added to the system just before closure. The substrate contained in the small test tubes was mixed with the remainder of the contents by tipping the flasks several times; they were then placed in a metabolic shaker at 25 C for 10 min. The reactions were stopped and CO_2 released by addition of lactic acid (0.1 ml, 85%) by injection through the rubber septa. After 30 min of additional shaking, the center wells were removed, and the individual wells were transferred to Tritosol for scintiallation counting.

Quantitative Estimation of 9,10-Dibromopalmitoylcarnitine

To detect and measure this acylcarnitine ester in eluates from silicic acid columns, we used Dragendorff's reagent. Palmitoylcarnitine or its dibromo-derivative give precipitates with this reagent. These were pelleted by low speed centrifugation, the excess reagent was decanted and the precipitates washed with 1 N HCl. The drained precipitates were then dissolved in 2 ml of acetone and the absorbancy was measured at a wavelength of 375 nm. This assay gave a straight-line calibration curve over the concentration range of 0-3 μmol/2 ml.

RESULTS

Characterization of the Bromination Product

Because it has been shown that 2-bromo palmitic acid is a potent inhibitor of fatty acid oxidation (15,16), it was important to determine that the conditions we defined for bromination of palmitoleic acid did not introduce bromine at other than the 9,10-positions. When a solution of the product was examined by proton NMR spectroscopy, it yielded an integration pattern for $CH_3/CH_2/CH/COOH$ (1:11-12:2-3:1) which was consistent with specific bromination of the double bond. More specific evidence was obtained by MS, which showed (Fig. 1) a peak at m/e = 428, with peaks at P + 1 and P + 2, consistent with the molecular weight of the expected product and with the presence of not more than 2 bromine atoms per molecule. A series of fragment peaks was also consistent with the proposed structure (17,18).

Purity of the preparation was assessed by TLC on Silica Gel G plates. Separations in 1 dimension (neutral plates) gave a single spot with R_f = 0.75; 2 dimensional separations (basic plates) (19) also gave a single spot with R_f = 0.49 and 0.71 for the first and second dimensions, respectively.

Effect of Added [U-^{14}C]-DBPA on Mitochondrial Oxygen Uptake

When an ethanolic solution of dibromo palmitic acid was added to a suspension of mitochondria prior to coupling by carnitine + ATP, the quantity of oxygen taken up by mitochondrial respiration was not significantly increased over the control values, but the maximum rate of uptake was significantly reduced, as shown in Table I. That is, the quantity of oxygen consumed (expressed as natoms 0/mg protein) can be estimated by the area under the polarographic curve. If, in our experiments, one waited until the recording of cuvet oxygen concentration after a DBPA addition had returned to a stable reading, no significant effect of the addition could be detected. A return to the stable state did require a longer time than did the addition of ethanol blanks. Effect of DBPA on the maximum *rate* of oxygen uptake was more reliably measured in another way, by direct reading of a simultaneous tracing which recorded the first derivative of the cuvet oxygen concentration. The effects did not result from the solvent ethanol. Lack of measurable differences in oxygen uptake suggested no β-oxidation occurred. For this reason, mitochondria were incubated with uniformly labeled DBPA acid in completely closed vessels containing CO_2 traps, as already described. In no instance was the scintillation counting rate of the CO_2 traps significantly above background, from which we

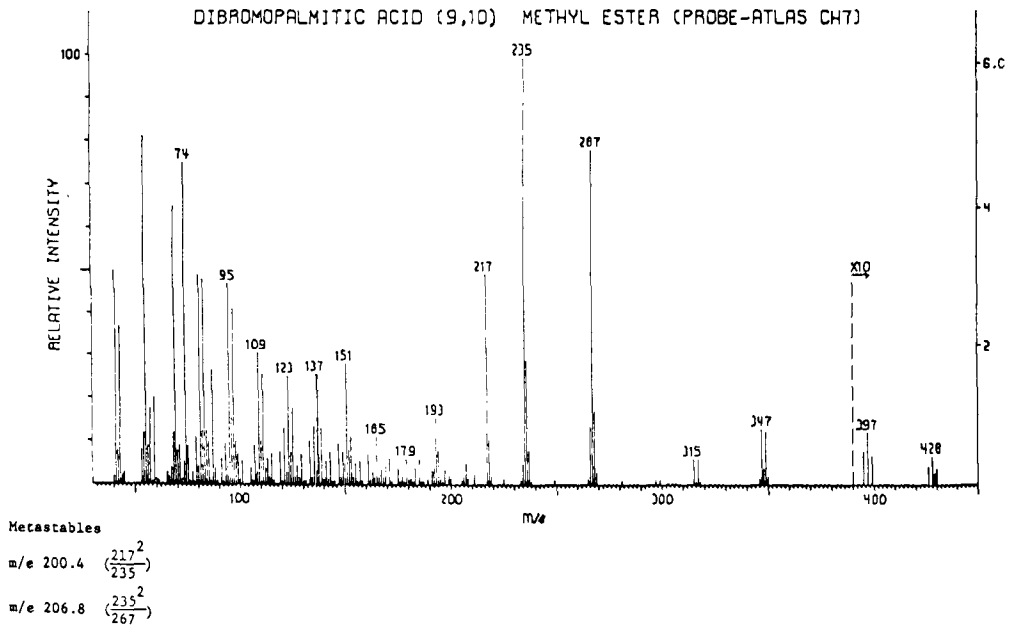

FIG. 1. Mass spectrogram of methyl (D,L)-threo-9,10-dibromopalmitate.

conclude β-oxidation had not occurred.

To determine the precise fate of the added DBPA, larger scale incubations were performed using a 10 ml polarographic cell. The entire cell content was collected, separated into a mitochondrial pellet and the supernatant suspension medium, and each fraction was subjected to lipid extraction as described. Recovery of added radioactivity is shown in Table II. In the course of these experiments, it was observed that the brominated fatty acid was more strongly adsorbed to the surface of the polarographic cell, constructed of methacrylate polymer, and to ordinary glassware than was palmitic acid. In order to remove the last traces of adsorbed material, it was necessary to wash the cell with a 1% bovine serum albumin solution. Similarly, in order to minimize losses of material during other manipulations, all glassware was treated for 24 hr with a solution containing the polarographic buffer, plus 10% lactic acid and 50 nmol/ml of unlabeled DBPA to saturate adsorption sites. With these precautions, it was possible to recover more than

TABLE I

Effect of DBPA[a], Added before Carnitine Recoupling, on Oxygen Uptake

DBPA added, (nmol/mg protein)	Oxygen taken up,[b] (natoms 0/mg protein)	Maximum rate of oxygen uptake,[c] (natoms 0/mg protein/min)
0	78 ± 18	106 ± 44
1.0	66 ± 24	58 ± 32
5.0	71 ± 24	65 ± 35
10.0	62 ± 40	68 ± 41
15.0	75 ± 29	63 ± 42
15-50	75 ± 29	---
EtOH[d]	88 ± 24	125 ± 70

[a](D,L)-threo-9,10-dibromopalmitic acid.

[b]Each value based on at least 6 separate experiments using 6 different mitochondrial preparations. Values cited as the means ± standard deviation of the mean for each entry. The experimental points are not significantly different ($p = 0.01$) from the control.

[c]Effect of added DBPA significantly different ($p = 0.01$) from the control.

[d]Ethanol added in amounts up to 5% of the total cell volume.

TABLE II

Recovery of Added [U-^{14}C] DBPA[a]

Fraction	[U-^{14}C] Content, CPM recovered	Percent radioactivity recovered
Supernatant	32,068	
Pellet	9,547	
Buffer wash of cell	2,664	
BSA wash of cell	1,521	
	45,800	
	46,360	98.8% of total added[b]
Pellet		
$CHCl_3$ phase	2,250	
CH_3OH-H_2O phase	5,850	
Residue	744	
	8,844	
	9,547	92.6% of pelleted ^{14}C
Supernatant + buffer wash		
$CHCl_3$ phase	12,148	
CH_3OH-H_2O phase	15,300	
BSA wash of separatory system	6,310	
	33,748	
	34,732	97.2% of total in initial supernatant + buffer wash

[a](D,L)-threo-9,10-dibromo palmitic acid.

[b]Total [U-^{14}C] DBPA added (sp act = 100 μCi/mmol) = 46,360 cpm.

98% of the added radioactivity during the separation of the mitochondrial pellet.

When the pelleted material was subjected to lipid extraction, the recovery was over 92% of that originally present in the pellet. The suspending medium was similarly extracted with a recovery of radioactivity ca. 97% of that initially present in the supernatant plus the buffer wash. These high recoveries were essential to attempts at characterization of the radioactive materials recovered from nanomolar amounts of starting material.

Radioactivity contained in extracts of pelleted material and of the supernatant was distributed between the chloroform and aqueous methanol phases. The chloroform-soluble materials were examined by silicic acid column chromatography as we have described. Results for the experiments with the pellet extract are shown in Figure 2, and Figure 3 gives results for the extract of the supernatant materials. Detection of the synthetic carnitine ester in column eluates was done using the spectrophotometric procedure outlined earlier, detection of the synthetic CoA ester was accomplished by specific absorbance of the adenine moiety, and detection of the free acid was done using the 1-^{14}C-label and scintillation counting. The identical lot of silicic acid was used to prepare both sets of columns. There is close agreement between the elution volumes of peaks in the experimental extracts and those in the known mixtures.

In experiments with the pellet extract, Solvent I removed 59.4% of the total applied radioactivity; Solvent II removed the remainder. Chromatography of known mixtures strongly supports the hypothesis that the 2 components could be characterized as unaltered dibromo palmitic acid (the substance responsible for the first peak) and as the CoA ester (the substance responsible for the second peak).

In experiments with the supernatant extract, results were more complicated. Only 58% of the total applied radioactivity could be recovered from the column eluates. Material was again located in 2 distinct peaks, both eluted with Solvent I. No additional radioactivity could be eluted, even through 100 ml of Solvents II and III were passed through the column. We have no explanation for the low elution recoveries in these experiments, which

were repeated several times. The congruence of the 2 curves supports the hypothesis that the second peak in the extract of the supernatant fraction was dibromopalmitoylcarnitine.

Recovery of free bromo-fatty acid from both fractions suggested activation of the acid might be a rate-limiting step in mitochondrial metabolism. Other evidence supported this possibility. Thus, thiokinase preparations from avocado mesocarp, according to Galliard and Stumpf (20) or from rat liver microsomes (21) gave no product with DBPA, although product was obtained in reasonable yields with normal palmitic acid.

Effect of Added CoA and Carnitine Esters on Mitochondrial Oxygen Uptake

Experiments were performed in which chemically synthesized esters were independently added to freshly coupled mitochondrial suspensions. Results of these experiments are shown in Figure 4. After recoupling by addition of carnitine, CoASH and ATP, there is a brief burst of respiration, indicated by the steep slope of the diminishing oxygen tension in the suspension. This subsides to a basal rate, at which point a 2 μl aliquot of 95% ethanol was added to indicate lack of appreciable effects of this solvent. Subsequent addition of an aliquot (20 nmol/mg protein) of palmitoylcarnitine (shown by the symbol, P.C.) induced a rapid burst of respiration, since acyl carnitines are readily metabolized by BAT mitochondria. The rate of oxygen uptake following substrate addition was 15 nmol·min^{-1}·mg protein^{-1}.

Curve B is a similar experiment in which, at the point indicated, an ethanolic solution of DBPC (20 nmol/mg protein) was added. This produced no discernible effects on oxygen uptake rate, nor did it affect the oxidation rate following substrate addition. This demonstrates that brominated palmitoylcarnitine was not oxidized, but neither did it act as a competitive inhibitor of translocating or β-oxidation enzymes. Analogous results were obtained when DBPCoA was added in place of the carnitine ester (data not reproduced here).

Curve C represents a similar experiment in which free DBPA (20 nmol/mg protein) was added in place of the esters. Immediately after the addition there was a distinct increase in oxidation rate. Since it had already been shown that the free acid was not subject to β-oxidation, we concluded that the stimulation of basal respiration was caused by an uncoupling effect of the free fatty acid. This was substantiated by observation that the oxidation rate of palmitoylcarnitine was increased to ca. 1.6 times the control rate. This observation agrees entirely with the effects of such known uncouplers as FCCP. In fact, when BAT mitochondria are oxidizing palmitoylcarnitine at the maximal rate, the effects of the brominated fatty acid and FCCP are quite similar, as shown in Figure 5.

Effect of Configuration of the Brominated Palmitic Acid

Addition of bromine to the double bond of palmitoleic acid proceeds by *trans*-addition

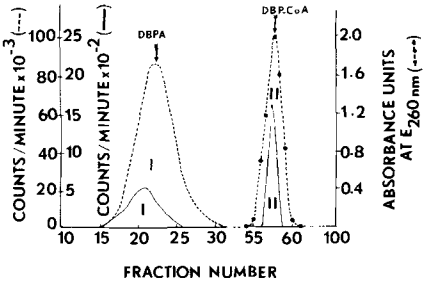

FIG. 2. Silicic acid column chromatogram of a mitochondrial pellet extract. Solid lines refer to chloroform-soluble material recovered from mitochondria previously treated with [U-^{14}C]DBPA (5.4 nmol/mg protein). Counting rate for these samples given by inner left hand ordinal scale. Dotted lines are a chromatogram of a mixture containing 10 μmol [1-^{14}C]DBPA plus 10 μmol DBPCoA applied directly to a similar column. Counting rate for the free acid indicated by outer left hand ordinal scale. The CoA ester was traced by absorbance measurements at 260 nm, with further identification by absorbance measurements at 232 nm before and after alkaline hydrolysis (data not shown here). Roman numerals I and II refer to eluting solvents, as described in the text.

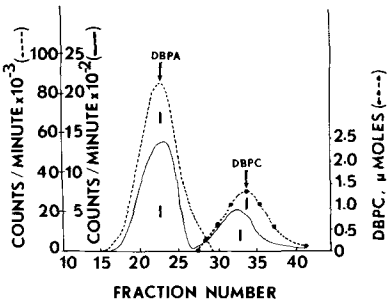

FIG. 3. Column chromatogram of suspension medium extract. Solid lines refer to chloroform-soluble material recovered from the suspension medium following removal of the mitochondrial pellet. Dotted lines refer to a mixture of 10 μmol of DBPA plus 10 μmol DBPC applied to a similar column. The carnitine ester was assayed by absorbance at 375 nm of an acetone solution of the complex formed with Dragendorff's reagent, as detailed in the text.

(22). Palmitelaidic acid was brominated under identical conditions, which should have produced D,L-erythro-9,10-dibromo palmitic acid. When samples of this material were added to mitochondria as already described, and compared to the effects of the threo compound, the results were indistinguishable; that is, no evidence for β-oxidation could be observed, and the addition uncoupled palmitoylcarnitine oxidation. For these reasons, efforts to measure carnitine or CoA ester formation with the erythro-dibromo compound did not seem justified. On the same basis, no effort has been made to resolve the brominated compounds into their optical isomers. It seemed unlikely that either isomer would compete with the other on the basis of current information.

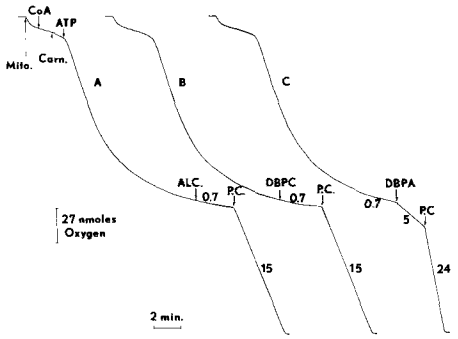

FIG. 4. Effect of added DBPA or DBPC on respiration of brown adipose mitochondria. The polarographic cell contained 1.5 mg mitochondrial protein suspended in 1 ml of buffer. Coupling of the mitochondria was induced by addition of K-ATP (2.5 mM), Coenzyme A (5 nM) and D,L-carnitine (3.3 μM). Additions of these reagents were as indicated at the upper left of the tracings. Trace A described a control experiment in which 2 μl of ethanol (ALC) was added, followed by an aqueous solution of palmitoylcarnitine (P.C.) (20 nmol/mg protein). Trace B is from a similar experiment in which an ethanol solution of (DBPC) (20 nmol/mg protein) was added. The last addition was palmitoylcarnitine, as in trace A. Trace C is from an experiment in which DBPA (20 nmol/mg protein) was added after mitochondrial coupling. Other conditions were as just described. Figures in the lower right represent the rate of oxygen uptake (nmol/min/mg protein) caused by oxidation of the palmitoylcarnitine.

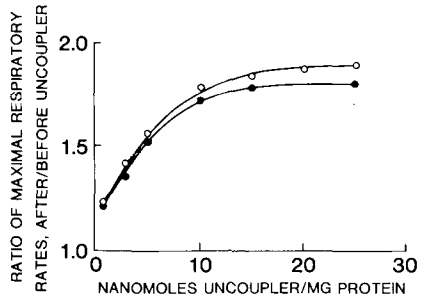

FIG. 5. Comparative effectiveness of dibromopalmitic acid and FCCP as uncouplers of oxidative phosphorylation during oxidation of palmitoylcarnitine by brown adipose mitochondria. Release of respiratory control increased maximal oxidation rates to approximately the same extent with either uncoupler. Open circles represent addition of FCCP, closed circles represent addition of DBPA. Each point is the mean of not less than 6 determinations, each involving 0.75-1.0 mg mitochondrial protein in a final volume of 0.91 ml.

DISCUSSION

Reports already mentioned on effects of feeding brominated oils lead to the conclusions that brominated fatty acids may be absorbed, that they are not readily oxidized and that they persist in tissue lipids for some time after brominated oil feeding is discontinued. These observations are reminiscent of similar observations following the feeding of *trans* mono- or polyenoic acids (23,24), although with *trans* unsaturated compounds it has been established that rat liver mitochondria have the enzymatic machinery to slowly effect total oxidation (25).

Results presented here are consistent with earlier evidence; dibromoacyl CoA esters are readily formed as a major metabolite of brominated fatty acids. Indeed, since the equilibrium constant for acyl exchange between CoA and carnitine esters is essentially unity (26), one might surmise that in intact tissues there would be a ready supply of activated bromoacyl groups available for incorporation into triglycerides or phosphatides (27) and that virtually all of the activated bromoacyl groups would funnel into those end products. It is also clear that DBPC does not act as a competitive inhibitor of acyl group transport or β-oxidation, since palmitoylcarnitine is quite readily transported and oxidized even in the presence of the brominated analogs.

Examination of molecular models of DBPA shows that introduction of the bulky bromine atoms produces several changes in molecular architecture, compared to palmitic, palmitoleic or palmitelaidic acids. First, there is hindrance of free rotation about the 9,10 carbon-carbon bond. Second, bromination introduces a considerable increase in electron density in the center of the hydrocarbon chain. As a consequence of bromination, the hydrocarbon chain becomes more straightened, since the bend resulting from the double bond has been eliminiated. Bromination also appears to minimize molecular association, since dibromo palmitic acid is a liquid at room temperature.

Thus, this molecule has the properties of both saturated and unsaturated acids.

It is not possible from the present evidence to determine how any of the possibilities described contributes to prevention of β-oxidation; neither is it possible to decide if the block is caused primarily by inability to transport the bromoacyl group into the matrix space, via the acylcarnitine-CoA transferase II, or by unacceptability of internalized bromoacyl groups as a substrate for any of the β-oxidation enzymes. Preliminary evidence suggests the problem is a matter of transport, but elucidation of these questions requires more study.

Finally, it should be pointed out that the properties of dibromo palmitic acid may make it a useful tool in examining the effect of changed lipid composition on membrane-bound enzyme function, or as an additional means of altering membrane fluidity.

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Effect of Ethanol Administration on Fatty Acid Desaturation

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ABSTRACT

The effect of ethanol on the fatty acid desaturation by rat liver has been studied using liquid diets of different composition. Acute ethanol administration increased triacylglycerols of total liver lipids, but did not modify significantly the lipidic composition of microsomes. The Δ6 and Δ5 desaturases were inhibited by ethanol whereas the Δ9 desaturase and fatty acid synthetase were apparently modified only by diet composition. NADH-cytochrome (cyt.) c reductase was partially inhibited, whereas NADH-cyt. b_5 reductase remained practically unaltered and NADPH-cyt. c reductase activity was enhanced. Decreased electrons supplied by the microsomal cyt. b_5 electron transport chain would not be the reason for the inhibition of Δ6 and Δ5 desaturases by ethanol.

INTRODUCTION

In animals, the desaturation of fatty acids occurs in the microsomal membrane. This reaction requires 3 amphipatic proteins embedded in the microsomal membrane: NADH cytochrome (cyt.) b_5 reductase, cyt. b_5 and the desaturase (1). There are different desaturases and their activity is related to dietary conditions. A diet high in carbohydrates increases the Δ9 desaturase, whereas a high-protein diet increases Δ5 and Δ6 desaturases and a fat-free diet increases both Δ9 and Δ6 desaturases (2).

Ethanol administration to rats produces fatty liver via accumulated fatty acids in hepatic triacylglycerols. The origin and extent of accumulation depend on dosage, period of administration and kind of diet (3,4). Liver triacylglycerols contain fatty acids provided by the fat depots, the diet or by endogenous synthesis (5).

Ethanol is oxidized to acetaldehyde and acetate through the cytosolic NADH dependent alcohol dehydrogenase (EC 1.1.1.1.) and aldehyde dehydrogenase (EC 1.2.1.3.). The oxidation of NADH has been considered the rate-limiting factor for this oxidation in vivo (6). However, the microsomal ethanol oxidizing system (MEOS) has been shown to contribute significantly to ethanol oxidation (7,8) and a role for catalase also has been suggested (9).

Since ethanol produces a fatty liver and microsomes are involved in both the biosynthesis of unsaturated fatty acids and alcohol oxidation, it is relevant to explore whether ethanol may modify (a) the lipid composition of liver and microsomes, and (b) the fatty acid desaturation reaction and some related enzymatic activities, such as NADH cyt. c reductase, NADPH cyt. c reductase, NADH ferricyanide reductase and fatty acid synthetase.

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MATERIALS AND METHODS

[1-^{14}C]Palmitic acid (55 mCi/mmol), [1-^{14}C]linoleic acid (50 mCi/mmol) and [1-^{14}C]eicosa-8,11-14-trienoic acid (61 mCi/mmol) were provided by New England Nuclear, Boston, MA. Cyt. c was provided by Sigma Chem. Co., St. Louis, MO. Cofactors for desaturation reaction were provided by Boehringer Argentina, Buenos Aires, Argentina.

Diet Treatment

In the first experiment, 15 female Wistar rats of 150-180 g body weight were divided into 3 lots: the first received Purina chow ad *libitum*, the second received a hyperlipidic diet and the third a hyperlipidic diet plus ethanol (see Table I). The second and third lots received isocaloric and force-fed diets, which were administered as 12.5 Kcal/100 g body weight every 12 hr for 48 hr.

In a second experiment, 20 female Wistar rats of 150-180 g body weight were divided into 4 lots. Each lot received the diet described in Table I. The isocaloric liquid diets were fed to the rats as described for the first experiment. Lot 2 received the same diet as lot 1, but 36% of dextrine calories were substituted by ethanol. Lot 3 received a diet without ethanol that maintained the same ratios of carbohydrates, lipids and proteins as lot 2; lot 4 received a diet with ethanol, but with the same ratios of carbohydrates, lipids and proteins as lot 1.

Microsomes

After the treatment, the rats were killed by decapitation. Livers were immediately collected

and homogenized in a solution containing: 0.25 M sucrose, 0.15 M KCl, 62 mM phosphate buffer (pH 7) and 1.5 mM glutathione. The homogenate was centrifuged at 10,000 x g for 20 min, the pellet was discarded and the supernatant was centrifuged again at 110,000 x g for 60 min. The supernatant was discarded and the pellet containing the microsomes was suspended in the homogenizing solution.

Fatty Acid Desaturase Assay

Fatty acid desaturase was assayed using 120 nmol of palmitic acid, 90 nmol of linoleic acid and 100 nmol o eicosa-8,11,14-trienoic acid. The acids were incubated with 5 mg of microsomal protein at 35 C for 20 min. Under these conditions, the enzymes were saturated by the substrate. The solution contained: 0.25 M sucrose, 0.15 M KCl, 0.04 M phosphate buffer (pH 7), 1.5 mM glutathione, 0.04 M KF, 1.3 mM ATP, 0.06 mM CoA, 0.87 mM NADH, 5 mM $MgCl_2$ and 0.33 mM nicotinamide in a final volume of 1.6 ml. Reactions were stopped by the addition of 2 ml of 10% methanolic KOH. Fatty acids were saponified for 45 min at 80 C under nitrogen, then acidified and extracted 3 times with 2 ml of petroleum ether (30-40 C bp). They were sterified 30 min with 3 N HCl in methanol. Conversion was measured by gas liquid radio chromatography in a Packard apparatus (with proportional counter) as described elsewhere (10).

NADH Cytochrome B_5–Ferricyanide Reducase Activity

Ferricyanide reductase activity was assayed at 25 C by measuring the NADH oxidation at 340 nm. The reaction mixture contained 30 nmol of NADH, 70 nmol of potassium ferricyanide and 2-10 μg of microsomal protein in a final volume of 0.27 ml of 0.05 M Tris acetate (pH 8.1) and 1 mM ethylenediaminetetraacetate (EDTA). The absorption decrease at 340 nm was followed as a function of time. An extinction coefficient of 6.22 mM^{-1} x cm^{-1} was used.

NADH Cytochrome c–Reductase Activity

The reduction of cyt. c was measured at 550 nm using 20 nmol of cyt. c, 30 nmol of NADH and 2-10 μg of microsomal protein in a final volume of 0.27 ml of 0.05 M Tris acetate (pH 8.1) and 1 mM EDTA. The absorption increase at 550 nm was followed as a function of time. An extinction coefficient of 18.5 mM^{-1} x cm^{-1} was used.

The activity of NADPH cyt. c reductase was assayed similarly using 20 nmol of cyt. c, 30 nmol of NADPH and 2-10 μg of microsomal protein.

Fatty Acid Synthetase Activity

Fatty acid synthetase activity was assayed by the Bruckdorfer et al. method (11), in which NADPH oxidation was measured at 340 nm.

Lipid Composition

Liver and microsomal lipids were extracted by the Folch et al. procedure (12). Complex lipids were separated in Silica Gel G (20 x 20 cm plates 0.5 mm thick), with chloroform/methanol/water (65:25:4, v/v), whereas the simple lipids were separated with petroleum ether/ethyl ether/acetic acid (90:10:1, v/v).

Spots were developed by spraying the plates with H_2SO_4 (70% saturated with $Cr_2O_7K_2$) and then heating at 200 C for 30 min. They were quantified by photodensitometry with adequate reference standards (13).

RESULTS

When ethanol is included in the rat diet, an imbalance in fatty acid metabolism is produced

TABLE I

Percentage Caloric Composition of the Diets

	Experiment 1			Experiment 2			
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 4
Carbohydrate (Dextrine)		40	4	53	17	26.6	34
Fat (Oil mixture)[a]	Purina chow	41	41	25	25	39	16
Protein (Casein)		19	19	22	22	34.4	14
Ethanol	---	---	36	---	36	---	36

[a]16:0 = 11.8%; 16.1 = 0.3; 18:0 = 3.1; 18:1 = 27.6; 18:2 = 57.2.

and the animal develops a fatty liver. This can be produced either by repeated administration of low doses of ethanol or by a large single dose (3,4). Hyperlipidic diets contribute to this fatty liver production. In the first experiment, the effect of repeated doses of ethanol for 48 hr was studied on rats receiving a hyperlipidic diet (Table I). The compositions of liver lipids and liver microsomes were determined (Table II). The activities of Δ5, Δ6 and Δ9 desaturases, the NADH-cyt. c reductase, NADH-ferricyanide reductase and fatty acid synthetase were measured (Table III).

In the first place, it was found that a change in the diet from Purina chow to an hyperlipidic diet evokes only minor changes in the lipid composition of the liver—an unimportant decrease in triacylglycerols. A decreased phosphatidylcholine (PC) and an increased phosphatidylethanolamine (PE) were shown in the microsomes. The Δ5 and Δ6 desaturases were not significantly altered, and the NADH-ferricyanide reductase and NADH-cyt. c reductase were only decreased to a minor extent, whereas the activities of the Δ9 desaturase and fatty acid synthetase were drastically

TABLE II

Percentage Lipid Composition in Experiment 1

		Liver			Microsome		
		Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
Diacylglycerols		1.3	1.0	1.2	1.0	0.9	1.2
Cholesterol		8.2	5.3	4.2	3.6	3.7	3.2
Free fatty acids		1.1	3.1	2.0	2.1	1.6	2.2
Triacylglycerols		19.0	12.9	35.5	5.5	6.0	7.2
Cholesterol esters		5.5	7.5	7.1	1.4	3.7	3.6
Lysophospholipids		5.8	10.1	4.1	13.5	12.3	10.2
Phosphatidylcholine		37.6	36.2	23.7	52.9	40.9	42.1
Phosphatidylethanolamine		21.7	23.9	22.2	20.1	30.8	30.4
% Caloric dietary ratios[a]	C	Purina	40	4	Purina	40	4
	F	chow	41	41	chow	41	41
	P		19	19		19	19
	E		---	36		---	36

[a]C = carbohydrate; F = fat; P = protein; E = ethanol.

TABLE III

Enzymatic Activities in Experiment 1

Enzymes		Lot 1	Lot 2	Lot 3
Δ9 Desaturase (% conversion)		53.0 ± 3.2[a]	8.2 ± 0.5	7.1 ± 0.3
Δ6 Desaturase (% conversion)		23.3 ± 1.5	23.3 ± 1.9	14.1 ± 1.1
Δ5 Desaturase (% conversion)		19.9 ± 3.3	17.2 ± 0.9	12.3 ± 1.8
Fatty acid synthetase ($nmol/min^{-1}/mg^{-1}$)		37.6 ± 4.7	6.0 ± 0.4	5.8 ± 0.7
NADH cyt.c reductase ($\mu mol/min^{-1}/mg^{-1}$)		0.87 ± 0.04	0.46 ± 0.01	0.30 ± 0.01
NADH cyt.b_5 ferrireductase ($\mu mol/min^{-1}/mg^{-1}$)		2.51 ± 0.08	2.02 ± 0.08	1.86 ± 0.11
% Caloric dietary ratios[b]	C	Purina	40	4
	F	chow	41	41
	P		19	19
	E		---	36

[a]Results represent the mean of 5 samples ± SEM.

[b]C = carbohydrate; F = fat; P = protein; E = ethanol.

diminished.

When ethanol replaced 36% of the calories provided by the carbohydrates of the hyperlipidic diet, the result was the typical increase of triacylglycerols in the total liver lipids. Triacylglycerols are minor components of the microsomes and were not modified (Table II). Δ6 Desaturase, Δ5 desaturase and the NADH-cyt. c reductase were significantly inhibited, but the activities of the other enzymes were not modified significantly.

These results would suggest ethanol decreases the activity of the Δ5 and Δ6 desaturation of fatty acids and the microsomal electron transport involved in the fatty acid desaturation, since the NADH-cyt. c reductase was inhibited. So, the steps that would be altered are the Δ5 and Δ6 desaturases and the electron transport from the cyt. b_5 reductase to the cyt. b_5 (Table III).

However, since 36% of the calories provided by carbohydrates in the hyperlipidic diet (lot 2) were replaced by ethanol (lot 3) one might speculate that the decrease of the Δ6 desaturase and NADH-cyt. c reductase activities were evoked by a decreased percentage of dietary carbohydrates. In order to discount this possibility, a second experiment was programmed (Table I): a carbohydrate-rich diet was tested (lot 1) and compared to a similar diet in which 36% of the carbohydrate calories were replaced by ethanol (lot 2). Lot 3 received a diet without ethanol which maintained the same ratios of carbohydrates, lipids and proteins as lot 2; lot 4 received a diet with ethanol, but with the same ratios of carbohydrates, lipids and proteins as lot 1.

Total liver lipid composition showed variations in triacylglycerol content as follows: lot 1, 11.7%; lot 2, 26.5%; lot 3, 6.8%; and lot 4, 23.4%. The microsomal liver lipid composition showed no significant variation among the 4 lots and it was similar to the composition shown in Table II. The unsaturated fatty acid:saturated fatty acid ratio in microsomal fatty acid composition also showed no significant variation.

The effects of the diets on the enzyme activities is summarized in Table IV. The comparison of lot 1 with lot 2, where 36% of dextrine calories were replaced by ethanol, showed an inhibition of the 3 desaturases (Δ9, Δ6 and Δ5), the fatty acid synthetase and the NADH-cyt. c reductase, whereas the NADH-ferricyanide reductase remained unmodified. In this experiment, NADPH-cyt. c reductase, an enzyme involved in the transport of electrons to cyt. P_{450}, was also measured and we found the activity was enhanced.

However, when the effect of ethanol feeding (lot 2) was compared to rats fed on ethanol-free diet (lot 3) in which the proportion of carbohydrates, lipids and proteins remained constant, ethanol only inhibited the Δ6 and Δ5 desaturases; the Δ9 desaturase and the fatty acid synthetase were unmodified. For the reductases tested, only the NADH-cyt. c and the NADH-cyt. c reductase were changed by ethanol.

TABLE IV

Enzymatic Activities in Experiment 2

Enzymes		Lot 1	Lot 2	Lot 3	Lot 4
Δ9 Desaturase (% conversion)		16.2 ± 1.1[a]	9.6 ± 0.9	9.7 ± 0.8	44.5 ± 4.1
Δ6 Desaturase (% conversion)		18.0 ± 1.2	9.9 ± 0.8	18.3 ± 0.6	11.1 ± 1.0
Δ5 Desaturase (% conversion)		27.6 ± 0.9	16.7 ± 2.4	32.0 ± 0.6	17.5 ± 1.5
Fatty acid synthetase ($nmol/min^{-1}/mg^{-1}$)		40.7 ± 2.4	24.8 ± 3.6	23.6 ± 1.9	71.0 ± 3.2
NADH cyt.c reductase ($\mu mol/min^{-1}/mg^{-1}$)		0.6 ± 0.06	0.36 ± 0.01	0.60 ± 0.06	0.43 ± 0.03
NADH cyt.b_5 ferrireductase ($\mu mol/min^{-1}/mg^{-1}$)		2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
NADPH cyt.c reductase ($nmol/min^{-1}/mg^{-1}$)		52.6 ± 0.3	77.3 ± 1.5	52.9 ± 4.1	76.4 ± 11.9
Percent caloric dietary ratios[b]	C	53	17	26.6	34
	F	25	25	39	16
	P	22	22	34.4	14
	E	---	36	---	36

[a]Results represent the mean of 5 samples ± SEM.

[b]C = carbohydrate; F = fat; P = protein; E = ethanol.

Apparently, the specific effect of ethanol is produced only in the Δ6 and Δ5 fatty acid desaturases and the NADPH-cyt. c and NADH-cyt. c reductase. The comparison of the decreased fatty acid synthetase and Δ9 desaturase activity of lot 2 with lot 1 indicates there is no specific ethanol effect, since the activity of both enzymes shows a similar decay when lots 3 and 1 are compared. Ethanol was not given to the rats in lots 1 and 3, but the diet composition was changed. The decreased Δ9 desaturase and fatty acid synthetase could result from decreased carbohydrates in the diet (14) or from an increased polyunsaturated lipid content (15), or from a combined effect.

The comparison of lot 2 with lot 4 in Table IV supports this last interpretation. Both groups of rats received, in this case, the same caloric intake of alcohol, but lot 4 was fed a diet richer in carbohydrates and lower in polyunsaturated lipid than the lot 2 diet.

DISCUSSION

The results of the experiments described show the modification of lipid metabolism produced by ethanol addition. Ethanol also increases liver triacylglycerols.

When rats are given a low-fat diet and ethanol is included, the liver fatty acid biosynthesis is enhanced (4,5). However, it has been shown that fatty acid synthetase and Δ9 desaturase activities respond to diet changes and that carbohydrates activate both enzymes (2,14-16). Therefore, the results of Table IV must be analyzed considering the lower lipid and higher carbohydrate content of the diet in lot 4 which cause increased activities of fatty acid synthetase and Δ9 desaturase when compared to lot 2. Also, administering ethanol, fewer carbohydrates and the same lipid and protein content to lot 2 results in lower synthetase and Δ9 desaturase activities than in lot 1. Under our experimental conditions than the activities of these enzymes are probably more dependent on the diet components than on ethanol ingestion.

Ethanol decreases the biosynthesis of polyunsaturated fatty acids. This effect is produced by (a) a decreases Δ6 desaturase activity that converts linoleic acid to γ-linolenic acid, and (b) a decreased Δ5 fatty acid desaturation that converts eicosa-8,11-14-trienoic acid to arachidonic acid. The decreased Δ6 and Δ5 desaturase activity is apparently caused by ethanol and not by a different composition of the diet for the following reasons: first, it is known that carbohydrates decrease the Δ6 desaturase (17) and that they may not modify the Δ5 desaturase (10). However, if we compare lots 2 and 1 of Table IV, we see the decreased carbohydrates (lot 2) neither, in this case evoke, an increased Δ6 desaturase nor change the Δ5 desaturase. On the contrary, we found that both enzyme levels decreased and suggest this effect results from the ethanol administration to lot 2. Second, proteins are known to increase the Δ6 desaturase level (17). In spite of the dietary proteins provided consistently to lots 1 and 2 decreases in Δ5 and Δ6 desaturases resulted.

The decreased Δ6 and Δ5 desaturase activities are probably not caused by a change in the lipid composition of the microsomal membrane. (The fatty acid desaturases and the electron transport system involved in the fatty acid desaturation are embedded in the membrane). (Table II).

Ethanol feeding induces a small but significant decrease in the microsomal electron transport activity involved in fatty acid desaturation. The electron transport from NADH to cyt. b_5 is significantly reduced; the specific decrease is not, however, produced by the cytochrome b_5 reductase activity but by the flux of electrons from the flavoprotein to the cyt. b_5. Moreover, the NADPH-cyt. c reductase activity of the microsomes was enhanced by the alcohol (Table IV) and this enzyme is linked mainly to the cytochrome P_{450} and MEOS systems which oxidize ethanol (7,8,18). Therefore, we see (a) a decreased electron flux to the cytochrome b_5 and polyunsaturated fatty acid desaturation and (b) an increased electron flux to the microsomal system involved in the oxidation of alcohol.

It is improbable that the decreased electron flux to the cytochrome b_5, shown in Tables III and IV, might induce a decreased Δ6 and Δ5 desaturation of fatty acid for the following reasons: first, the same electron transport system is used for the Δ9, Δ6 and Δ5 desaturation (19) and the Δ9 desaturation of fatty acids was not diminished by ethanol (Tables III and IV); Second, the supply of electrons by the NADH-cyt. b_5 system is so high that, provided sufficient NADH is present, it can easily fulfill the requirements of fatty acid desaturation (20-22). It has been experimentally shown that even after 99% inhibition of NADH-cyt. b_5 reductase activity, there are enough electrons to account for 80% of the desaturase activity (20,21). Finally, NADPH may provide electrons to cyt. b_5 and fatty acid desaturase by a link through the NADPH-cyt. c reductase (18). The fatty acid desaturase is generally the rate-limiting step of the fatty acid desaturation system, since it has the lowest turnover number

(20,22). These results would therefore suggest that alcohol evokes a specific effect on the Δ6 and Δ5 desaturation of fatty acids.

These experiments demonstrate again that the Δ9 desaturase follows a pattern of activity control different from the Δ6 and Δ5 desaturases—a thesis already stated by us in previous works (2). Moreover, the Δ9 desaturase and fatty acid synthesis would not be modified by ethanol under the conditions of this experiment; they follow a behavior similar sequence to that caused by changes in the diet. We suggest they may be linked as a unit by a common mechanism, a theory already proposed by Jeffcoat and James (23).

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METHODS

A Single-Step Method for Determination of the Specific Radioactivity of Lipids

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ABSTRACT

The use of a liquid scintillation counter to measure both the mass and the radioactivity content of charred ^{14}C-labeled lipid bands from thin layer chromatoplates has been evaluated. Following lipid mass determination from a measurement of the external standard channels ratio, a suitable choice of counting parameters enabled a reproducible and efficient ^{14}C count to be obtained over virtually the whole range of lipid concentrations tested. Although the charring procedure resulted in some loss of radioactivity, the efficiency of counting remained high enough for accurate dpm measurements.

INTRODUCTION

To consider the use of a liquid scintillation counter for purposes other than the quantification of radioactivity is unusual. However, as recently shown, its use in the laboratory can be extended considerably outside this function into areas of analysis with which it is not normally associated. As a result, this normally specialized piece of equipment can be used for quantification in a range of colorimetric and ultraviolet (UV) absorption analyses (1). More recently, the liquid scintillation counter has been applied to the quantification of lipid mass through the densitometric measurement of charred lipid bands from thin layer chromatoplates (2). The method is based on the ability to measure the amount of suspended, charred material through a reduction in the value for the external standard channels ratio (an external standard is incorporated into modern liquid scintillation counters in order to measure the extent of quenching). Among the many suggested advantages for this technique over existing methods was the possibility that, in circumstances where radioactive lipids were being investigated, specific activity (sp act) data could be provided in a single analytical step, a feature not possible with other techniques for lipid quantification. This aspect of the method has now been investigated.

EXPERIMENTAL PROCEDURE

Standard solutions of pure glycerol tri[1-^{14}C] palmitate, [1-^{14}C]palmitic acid and [4-^{14}C] cholesterol (Radiochemical Centre, Amersham, England) were made up to provide varying amounts (ranging from 0.01-1.0 mg) of lipid of known dpm and were separated as small (ca. 8 cm) discrete bands on thin layer chromatoplates of Silica Gel G (E. Merck, Darmstadt, Germany). The subsequent charring in situ and suspension of each lipid band as a thixotropic gel within a standard 20 ml glass scintillation vial was as described previously (2). Each sample was counted first for 1 min in the external standard channels ratio mode of a Model 2425 liquid scintillation counter (Packard Instruments Ltd., Downers Grove, IL) and then for a suitable period of time at a spectrometer setting appropriate for the quantification of ^{14}C radioactivity in gels. The value obtained for the external standard channels ratio was then interpolated into (a) a series of previously derived curves for concentration of lipid vs external standard channels ratio to determine the concentration of lipid and (b) a series of previously derived curves for efficiency of ^{14}C count vs external standard channels ratio to determine dpm ^{14}C. The ability to derive accurate sp act data over the complete dynamic range of the method was then evaluated.

RESULTS AND DISCUSSION

Figure 1 shows the calibration curves obtained for mass of lipid and efficiency of ^{14}C counting vs the external standard channels ratio. The curves relating the mass of lipid to the external standard channels ratio were similar to those obtained previously and demonstrated a range for the accurate determination of mass from 0.01-1 mg lipid. Although the response to charring (as measured by the change in the external standard channels ratio) for the lipid classes was similar, a wider dis-

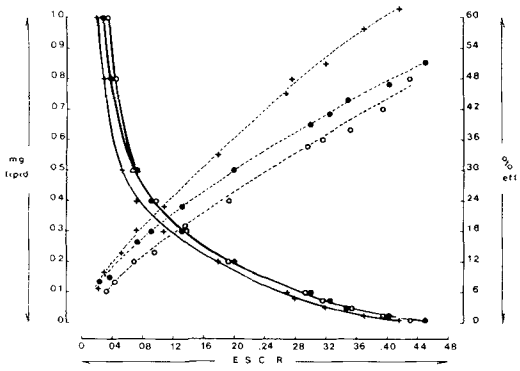

FIG. 1. Calibration curves relating the external standard channels ratio (ESCR) to (a) the amount of lipid applied to the thin layer plates: ●——● tripalmitin; ○——○ palmitic acid; +——+ cholesterol, and (b) the ^{14}C counting efficiency: ●------● tripalmitin; ○------○ palmitic acid; +------+ cholesterol. Each point is the mean of 6 observations. In no case was the standard error greater than ± 3% of the mean value.

parity was evident with regard to efficiency of ^{14}C counting. The maximum efficiencies of counting, i.e., those obtained at 0.01 mg of charred lipid, were 51% (S.E. ± 0.6), 49% (S.E. ± 0.7) and 63% (S.E. ± 0.7) for tripalmitin, palmitic acid and cholesterol, respectively. In the absence of any spray reagent or charring, the efficiency of counting of the lipid bands was 81% (S.E. ± 0.4). The presence of the small amount of charred material from 0.01 mg lipid accounted for only a small proportion of this decrease in counting efficiency; loss of radioactivity from the factors involved in the charring procedure were clearly the major reason for the reduction in counting efficiency. Thus, where standard amounts of virtually carrier-free ^{14}C-labeled lipids were applied to thin layer chromatoplates, which were sprayed and charred, counting efficiencies of only 53% (S.E. ± 0.8), 51% (S.E. ± 0.8) and 70% (S.E. ± 1.4) were obtained for the tripalmitin, palmitic acid and cholesterol, respectively, compared with counting efficiencies of 74% (S.E. ± 1.1), 76% (S.E. ± 1.2) and 76% (S.E. ± 1.3), respectively, when the bands were sprayed but not charred. Reproducibility and accuracy of the curves for the external standard channels ratio vs ^{14}C counting efficiency were high over the full range of the lipid concentrations; in the absence of any major alteration to the overall analytical technique, adjustments to the quench correction curves were unnecessary. However, as a result of the presence of large concentrations of charred material, extensive quenching (efficiencies of 10% or less) was observed at concentrations of 0.8-1.0 mg lipid. Comparisons of actual and derived values for the radioactivity content of a series of known lipid standards are given in Table I. Accurate mass determinations were possible over the complete range (0.01-1.0 mg) of lipid but the levels of quenching associated with 1.0 mg lipid caused somewhat larger errors in the dpm ^{14}C determination. Whether the error introduced into the determination of absolute quantities of

TABLE I

A Comparison of the Actual Amounts (dpm x 10^{-3}) of ^{14}C Radioactivity Applied to Thin Layer Plates with That Estimated from Quench Correction Curves [a]

		Tripalmitin	Palmitic acid	Cholesterol
dpm (x 10^{-3}) Applied to plate		39.8	25.8	17.9
Calculated dpm (x 10^{-3}) at different lipid concentrations				
Lipid conc. (mg)	0.01	39.9 ± 0.4	25.2 ± 0.4	18.1 ± 0.2
	0.025	40.0 ± 0.4	24.7 ± 0.3	18.4 ± 0.2
	0.05	40.2 ± 0.7	24.5 ± 0.4	18.0 ± 0.2
	0.075	39.7 ± 0.5	24.6 ± 0.3	17.8 ± 0.2
	0.1	40.9 ± 0.4	26.3 ± 0.3	18.5 ± 0.2
	0.2	40.2 ± 0.9	25.0 ± 0.3	17.9 ± 0.3
	0.3	40.2 ± 0.9	25.2 ± 0.7	17.6 ± 0.1
	0.4	39.9 ± 1.1	26.4 ± 0.5	18.2 ± 0.3
	0.5	40.1 ± 0.8	27.8 ± 0.9	18.4 ± 0.3
	0.8	38.4 ± 2.0	27.9 ± 0.9	18.3 ± 0.5
	1.0	42.3 ± 1.4	27.5 ± 0.5	16.7 ± 1.0

[a]Each result is the mean ± SD of six determinations.

radioactivity from counting efficiencies of ca. 10% exceed acceptable limits depends entirely upon the needs of the individual user and the limits of accuracy for the experiment but, as usual in radioactive determinations, they must be viewed with some caution. The ability of the method to provide accurate quantification of the radioactive content of the lipid classes from normal biological extracts was demonstrated by analyzing a sample of ^{14}C-labeled pig liver lipids. As determined by conventional means, the radioactivity contents of the triglyceride, unesterified fatty acid and free cholesterol fractions were 4.83×10^4, 2.78×10^4 and 1.13×10^4 dpm, respectively. Values obtained using the quench correction curves shown in Figure 1 for the lipid bands following charring were 4.81 (S.E. ± 0.07), 2.90 (S.E. ± 0.06) and 1.18 (S.E. ± 0.01) dpm, respectively.

These investigations serve to show that, providing separate quench correction curves are drawn up for individual lipid classes (a procedure which is normal, good laboratory practice), the suggested method for the quantification of lipid mass from thin layer chromatoplates following charring in situ does allow the accurate determination of ^{14}C radioactivity over a range of lipid concentrations that would be more than adequate to cover most practical thin layer chromatographic conditions. Its application to lipids containing isotopes of similar or greater emission energies to that of ^{14}C, e.g., ^{35}S or ^{32}P, is obvious. In the case of ^{3}H-labeled lipids, however, the combined effects of gel counting and quenching by the charred lipid material on the transmission of the considerably weaker photon emissions preclude the possibility of its use.

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COMMUNICATIONS

Lipids of Myelin, White Matter and Gray Matter in a Case of Generalized Deficiency of Cytochrome b_5 Reductase in Congenital Methemoglobinemia with Mental Retardation

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ABSTRACT

The lipid classes and fatty acid compositions of myelin, white matter and gray matter were analyzed in a case of generalized deficiency of cytochrome b_5 reductase in congenital methemoglobinemia with mental retardation. When compared with normal data, the percentage of 24:1 was considerably decreased and diminished unsaturation was observed in cerebrosides, whereas the sum of 24:0 and 24:1 was the same as in normals. The ratio of hydroxy fatty acids to total fatty acids in cerebrosides was low. The contents of cholesterol and phospholipids in white matter were reduced to 80% of the normal, whereas cerebroside was reduced to 48% of the normal.

INTRODUCTION

A generalized deficiency of cytochrome b_5 reductase is expected to produce a systemic impairment of the metabolic process linked to cytochrome b_5, such as fatty acid desaturation (1,2). The relationship between methemoglobinemia and nervous system function has been poorly understood, but it was reported that methemoglobinemia with neurological involvement might result from a generalized enzyme defect (3,4). Since the disease primarily affects the central nervous system, it was considered important to analyze the fatty acids of brain lipids in a methemoglobinemic patient.

MATERIALS AND METHODS

Brain material

A 33-month-old girl had died from congenital methemoglobinemia with mental retardation and bilateral athetoid syndrome caused by generalized deficiency of NADH cytochrome b_5 reductase. The brain was removed 4 hr after death and immediately frozen in dry ice and stored at -80 C.

Preparation of Myelin

The white matter was homogenized in 120 vol of 0.32 M sucrose as described earlier (5) and the myelin was isolated using the method described by Norton and Poduslo (6). The sucrose solutions were buffered with 10 mM Tris-HCl (pH 7.4).

Lipid Determinations

The lipid extraction and isolation of the individual phospholipids and determination of their fatty acid compositions have been described earlier (7-10).

Lipid phosphorus was determined according to Svennerholm and Vanier (9) and cholesterol according to Crawford (11). Sialic acid was quantified using the resorcinol procedure (12) and cerebrosides were isolated by preparative thin layer chromatography (TLC) and analyzed for galactose (12).

Controls

Control samples were obtained from an 11-year-old boy who had drowned. Sialic acid determination was done as a control. Other control data are cited from Svennerholm's laboratory data which were published in part (5). None of the subjects had any previous history of neurologic or psychiatric disease and all had normal body and brain weight. All brain samples were analyzed in the same laboratory using the same techniques as those used in the analysis of the patient's brain.

RESULTS AND DISCUSSION

There were decreases in the molar ratio of cholesterol to phospholipids in myelin but not in white matter; however, the ratio of cerebro-

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sides to phospholipids in the myelin and also white matter was reduced (Table I). The concentrations (μmol/g wet wt) of cholesterol and phospholipids in white matter were 80% of the normal, and cerebrosides were markedly reduced to 48% of the normal. Sialic acid content was higher in the white matter and less in the gray matter of the patient's brain as compared to control. The percentage of hydroxy fatty acid to total fatty acids in cerebrosides of white matter was 32.9%, which was very low compared to a normal value of 55% (13).

There was a slightly diminished proportion of 18:0 in ethanolamine phosphoglycerides (EPG) of gray matter, white matter and myelin (Table II). The n-3/n-6 ratios in EPG of white matter and gray matter were normal, but in myelin they were lower. The ratios of 18:0/18:1 in EPG and choline phosphoglycerides (CPG) were normal or slightly below normal.

There were considerable decreases in the proportion of 24:1 and slight increases in 24:0 in cerebrosides, sulfatide and sphingomyelin in myelin and white matter (Table III). The proportion of 25:1 was less in all fractions. The proportion of 26:1 in sphingomyelin, cerebrosides and sulfatide was higher in the myelin but less in the white matter when compared with normals. The ratio of 24:1/24:0 was reduced by one-half in myelin cerebrosides. The sum of 24:0 and 24:1 were almost the same as for normals. The concentrations of C_{22}-C_{26} fatty acids were normal or slightly below normal. However, considerably less unsaturation was observed in myelin cerebrosides.

The proportion of 24h:0 was normal and 24h:1 slightly below normal in white matter cerebrosides, and those of 24h:1 was less and 24h:0 greater in sulfatide (Table IV). There was no change in the concentration of C_{22}-C_{26} hydroxy fatty acids in both cerebrosides and sulfatide, but there was diminished unsaturation of hydroxy fatty acids in sulfatide. The changes in the hydroxy fatty acid composition of sulfatide showed the same trends as in the nonhydroxy fatty acids, whereas those in cerebrosides showed very slight change. Gerstl et al. (14) reported no change in the hydroxy fatty acid composition in myelin obtained from multiple sclerosis. Johnson and Shah (15) also did not find any change in hyperphenylalaninemia nor did Hirono and Wada (16) find any differences in folate deficiency.

In mammalian brain, 24h:0 is formed directly from 24:0, and 24:0 is formed by chain elongation of 16:0. The 24:1, on the other hand, is formed by chain elongation of

TABLE I

Brain Lipid Distribution

		Molar ratio to phospholipids		
		Cholesterol	Phospholipids	Cerebrosides
Myelin	Patient	0.95	1.00	0.29
	Normal 25-month[a]	1.17	1.00	0.37
White matter	Patient	1.04	1.00	0.20
	Normal 25-month[a]	1.04	1.00	0.29

		μmol/g wet wt			
		Cholesterol	Phospholipids	Cerebrosides	Sialic acid
White matter	Patient	93.70	90.01	18.4	1.23
	Normal 27-month[a]	118.5	112.1	38.3	
	Normal 11-year				0.96
Gray matter	Patient	26.50	43.63		2.98
	Normal 27-month[a]	24.8	39.4		
	Normal 11-year				3.45

[a]Normal data are cited from Svennerholm's laboratory data.

TABLE II

Fatty Acid Composition of Brain Glycerophosphatides[a]

	Ethanolamine phosphoglycerides			Choline phosphoglycerides		
	Gray matter	White matter	Myelin	Gray matter	White matter	Myelin
16:0	6.0	5.1	5.5	50.5	39.5	34.1
16:1	0.4	0.8	1.2	2.0	2.9	2.8
18:0	25.9	8.6	7.4	10.8	12.4	14.6
18:1(n-9)	9.1	33.7	35.2	25.0	39.0	38.9
18:2(n-6)	0.2	0.5	0.9	0.5	0.5	1.0
20:1(n-9)	0.4	4.6	4.7	0.5	0.9	1.2
20:3(n-9)			0.2			
20:3(n-6)	4.3	9.0	9.7	3.1	1.8	2.3
20:4(n-6)	17.0	11.8	11.2	5.2	2.1	3.4
22:4(n-6)	10.3	16.5	17.5	0.6	0.4	1.0
22:5(n-6)	2.6	1.7	1.7	0.2	0.2	
22:5(n-3)	0.6	0.7	0.6			
22:6(n-3)	23.3	7.0	4.4	1.5	0.4	0.6
(n-3)/(n-6)	0.694	0.194	0.121	0.156	0.080	0.077
18:0/18:1	2.85	0.29	0.21	0.43	0.32	0.38
Unsaturated	68.1	86.3	87.1	38.7	48.1	51.3

[a]Values are molar percentages of fatty acid methyl esters.

TABLE III

Fatty Acid Composition of Brain Sphingolipids[a]

	Sphingomyelin		Cerebrosides		Sulfatide	
	White matter	Myelin	White matter	Myelin	White matter	Myelin
16:0	4.8	5.7	1.0	1.8	2.1	3.4
16:1		0.5	0.1	0.3	0.3	0.4
18:0	40.8	30.7	6.1	5.6	4.6	4.2
18:1	0.9	4.0	1.4	1.5	2.7	2.4
20:0	1.3	1.2	0.8	0.7	0.6	0.8
22:0	3.3	4.5	3.7	3.4	3.7	3.8
22:1	0.7	1.2	0.6	0.5	0.5	0.6
23:0	2.2	2.3	4.8	3.8	3.5	3.0
24:0	10.9	13.7	25.7	29.1	24.7	25.6
24:1	26.4	24.4	36.8	27.5	36.4	30.3
25:0	1.8	2.6	6.7	6.2	4.7	4.8
25:1	2.0	1.8	3.3	3.7	3.4	3.4
26:0	0.8	1.6	1.9	3.6	2.4	3.8
26:1	4.1	5.9	7.3	12.3	10.6	13.7
22-26	52.2	57.9	90.8	90.1	89.9	88.8
Monoenes	34.1	37.8	49.5	45.8	53.9	50.8
24:1/24:0	2.42	1.78	1.43	0.94	1.47	1.18
24:0 + 24:1	37.3	38.1	62.5	56.6	61.1	55.9

[a]Values are molar percentages of the fatty acid methyl esters.

endogenous 18:1 (17,18). Bourre et al. (19) reported cerebrosides and sulfatide were drastically reduced to 8% of the normal, and they found marked decreases in both 24:0 and 24:1 in Pelizaues-Merzbacher disease. They concluded one aspect of the disease may be a defect in the synthesis of very long chain fatty acids. In our case, however, there was no impairment of chain elongation for 24:0 synthesis. Since the cytochrome b_5 reductase system is involved in fatty acid desaturation (20), myelin-related lipid abnormalities in our patient may have been a consequence of this enzyme deficiency.

TABLE IV

Composition of Hydroxy Acids in White Matter[a]

	Cerebrosides	Sulfatide
18h:0	0.82	1.03
20h:0	0.48	0.38
21h:0	0.19	
22h:0	8.99	7.81
22h:1	0.36	0.20
23h:0	10.19	7.69
23h:1	0.59	
24h:0	44.03	46.95
24h:1	15.10	15.03
25h:0	6.37	5.71
25h:1	2.48	2.55
26h:0	3.69	3.92
26h:1	6.69	8.73
22h-26h	98.5	98.6
Monoenes	25.2	26.5
24h:1/24h:0	0.34	0.32
24h:0 + 24h:1	59.1	62.0

[a]Values are molar percentages of the hydroxy acid methyl esters.

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The Effect of Dietary Fat on the Fatty Acid Composition of Lipids Secreted in Rats' Milk

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ABSTRACT

During pregnancy and lactation, female rats were fed diets containing either 28% partially hydrogenated marine oil (28MO), 2% arachis oil (2AO), or no fat (FF). Milk lipid composition was examined by gas chromatographic analysis of the gastric content of 10-day-old suckling pups. An increase to 45% in the milk content of long chain monoenoic acids, 18:1, 20:1 and 22:1, reflects the fatty acid composition of the marine oil. Milk fatty acids of medium chain length comprised 6%, 31% and 24% of total fatty acids in the (28MO), (2AO) and (FF) groups, respectively, suggesting that a high-fat diet (28MO) inhibits the lipid synthetic activity of mammary glands. The amount of dienoic C_{18}-acids (6%) in the group fed (28MO) containing no essential fatty acids (EFA) was similar to the amount of 18:2 in the group receiving a low-fat, EFA-rich diet (2AO). However, only half the dienoic acid from the milk of the (28MO)-fed animals was linoleic acid, which was most likely mobilized from fat depots.

INTRODUCTION

The main sources of milk lipids are dietary fat and fatty acids synthesized de novo by mammary glands (1-4). Thus, maternal diet may influence the nutritional quality of fat available to the sucklings (5-8). In this study, rat milk fatty acid composition was examined by gastric content analysis of 10-day-old suckling rats. Preintestinal lipolysis converts milk triglycerides into diglycerides and free fatty acids to a considerable extent (9,10); the evidence for gastric absorption is still questionable, however. The primary purpose of the experiments reported here was to study the effect of maternal dietary partially hydrogenated marine oil on the fatty acid composition of milk lipids consumed by the offspring.

MATERIALS AND METHODS

Rats (Wistar strain, K. Møllegård-Hansens Avlslab. A/S, Ll. Skensved, Denmark) reared on a semisynthetic, basic food (11) supplemented with 2% by wt arachis oil were mated, and from about the tenth day in the gestation period the female rats were dividied into 3 groups according to the experimental diets. Group 2AO, the control group, continued on arachis oil, 2% by wt (low-fat) equal to 2 energy percent of essential fatty acids (EFA). Group FF was fed a fat-free diet and group 28MO received the basic food supplemented with 28% by wt of partially hydrogenated marine oil (EFA-deficient, high-fat). The animals were fed the experimental diets ad libitum during late pregnancy (i.e., 11 days) and throughout lactation.

Lipid analyses were performed on the gastric content, which was immediately removed from decapitated 10-day-old sucklings and stored at -80 C prior to analysis. Four samples from each group were examined individually. Postfreezing weight was determined and freeze-drying was performed on each sample after suspension in a few ml of redistilled water. Lipids were extracted with 20 vol (relative to dry matter weight) of chloroform/methanol (2:1, v/v) with vigorous shaking by hand and successive filtration on a sintered glass filter (pore size 20-30 μm). No washing was performed to avoid possible loss of monoglycerides and fatty acids. The dry weight of the lipid extracts was determined on an electromicrobalance. Aliquots of extract containing ca. 15 mg of lipid were methylated (12) overnight by benzene/methanol-HCL/dimethoxypropane (7:2.5:0.5, v/v). After addition of redistilled water (10 ml), methyl esters were extracted with petroleum ether (100 ml), concentrated and redissolved in a known volume of petroleum ether. Temperature-programmed gas liquid chromatography (GLC) was carried out on a Perkin-Elmer gas chromatograph (F11) with steel columns (2 m x 3 mm) containing 15% diethyleneglycol succinate (DEGS) on Chromosorb W/AW. Fatty acid methyl esters were identified by comparing retention times with those of reference methyl esters (Nu-Chek-Prep, Elysian, MN) and further confirmed by GLC on methyl esters separated by $AgNO_3$-thin layer chromatography ($AgNO_3$-TLC) according to unsaturation of the fatty acids. Peak areas were integrated by an electronic integrator (Hewlett-Packard 3370B) and quantitated using an internal standard (pentadecanoic acid) added before methylation. All chemicals were analytical grade; solvents were redistilled.

RESULTS AND DISCUSSION

Lipids made up 57-67% of the dry matter of the gastric contents from all 3 dietary groups. The fatty acid composition of the milk lipids in the 3 groups is shown in Figure 1. Long chain monoenoic acids, 18:1, 20:1 and 22:1, from the dietary marine oil made up about half the fatty acids secreted in the milk of 28MO rats, indicating lipids absorbed from the intestine passed readily into the milk. During lactation, lipoprotein lipases in the mammary glands catalyze the liberation of fatty acids from blood lipoproteins (1,2,13), which are, in turn, incorporated into triglycerides and secreted into the milk. It has been shown (14,15) in humans that a high-fat diet suppresses the secretion of endogenously synthesized milk fatty acids of medium chain length. Similar results were obtained in this study with rats – evidenced by the fatty acid composition of the 28MO group milk (Fig. 1). Fatty acids of medium chain length (C_8 through C_{14}) comprised only 6%, whereas in the low-fat (2AO) and fat-free (FF) groups, medium chain acids made up 31% and 24%, respectively. Thus, regardless of a similar fat content in the dry matter, a marked difference in the medium chain length fatty acid percentage is seen. The regulatory mechanism of this shift in fatty acid composition cannot be deduced from this study. It may be an inhibitory effect of high-fat intake and blood content on the fatty acid or triglyceride synthesizing activity in mammary glands. Differences in the quality of the dietary fat (i.e., marine oil vs arachis oil) cannot be overlooked after comparing the groups in this experiment. However, similar results have been reported (14) with quantitative differences in the diet only.

EFAs were not included in the diets of group 28MO and group FF. The linoleic acid content of milk from group FF was reduced to 1.7%. In the 28MO group, the proportion of octadecadienoic acid was similar to the percentage (6%) found in the milk of the (2AO)-fed animals. Yet, about half of the dienoic C_{18}-acids were isomers, which were not completely identified; they did not, however, belong to the linoleic acid family as judged by GLC. Only trace amounts of another EFA (arachidonic acid) were detected in the milk from any of the 3 groups. The occurrence of EFAs at all in the milk from the non-EFA-fed animals indicates reserves, which can be mobilized as needed.

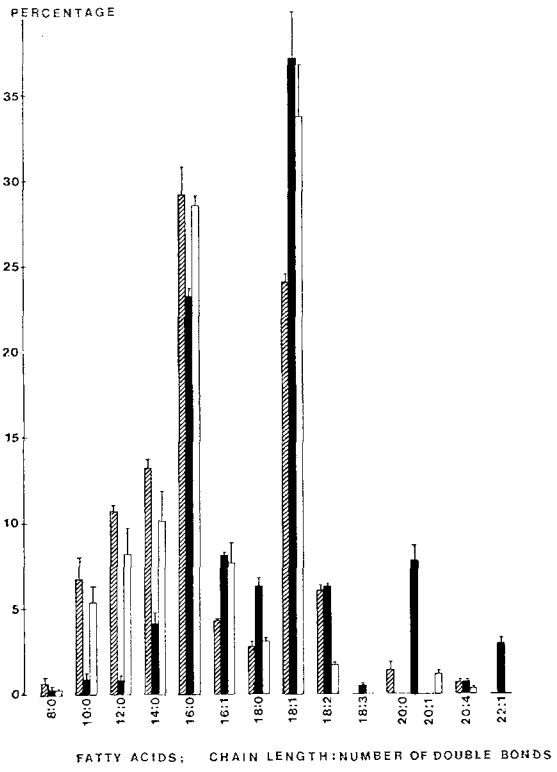

FIG. 1. Fatty acid distribution in milk lipids from rats fed 28% partially hydrogenated marine oil(■), 2% arachis oil (▨), or a fat-free diet (□). The bars represent means ± S.E. (N=4).

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ERRATUM

Figures 1 and 2 in "Prenatal Protein Depletion and 9, 6 and 5 Desaturases in the Rat" by Osvaldo Mercuri, María Elena De Tomás and Herminia Itarte (September 1979, page 823) were published in reverse order. They should have appeared as shown below.

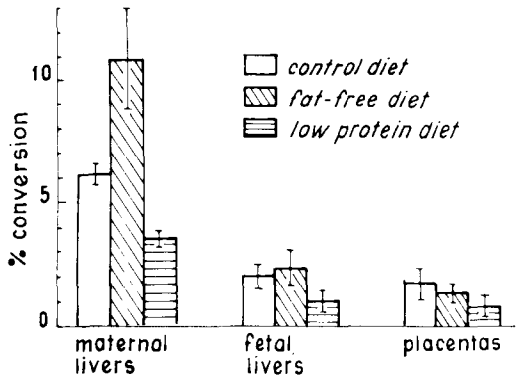

FIG. 1. Microsomal Δ9-desaturase activity from maternal and fetal livers and placentas from rats fed on different diets throughout the gestation. Each bar is the mean of five sample determinations ± standard deviation.

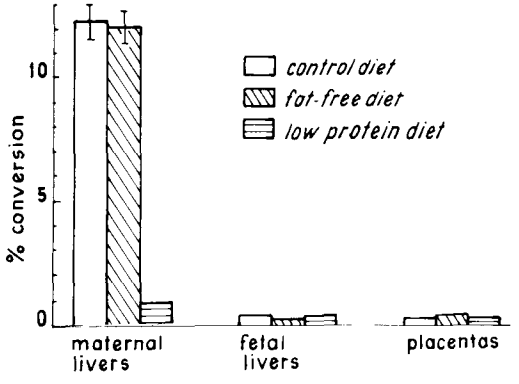

FIG. 2. Microsomal Δ6-desaturase activity from maternal and fetal livers and placentas from rats fed on different diets throughout the gestation. Each bar is the mean of five sample determinations ± standard deviation.

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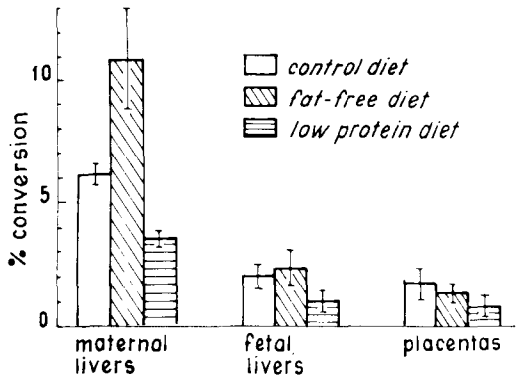

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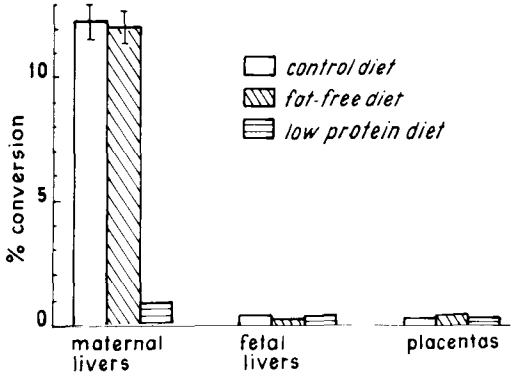

FIG. 2. Microsomal Δ6-desaturase activity from maternal and fetal livers and placentas from rats fed on different diets throughout the gestation. Each bar is the mean of five sample determinations ± standard deviation.

Molecular Species Composition of Phosphatidylcholines during the Development of the Avian Embryo Brain

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ABSTRACT

A comparative approach has been used to investigate the molecular species composition of phosphatidylcholine (PC) and its age variation throughout several developmental stages of chick and duck embryo brains. The brain PC consist of 15 major molecular species which do not undergo appreciable variation in their relative abundance either during embryonic development or between equivalent stages of maturation in the 2 avian species. In fact, a highly invariable molecular architecture of PC is shown in the developing organ. Molecular species containing saturated or monounsaturated fatty acids were dominant in all stages of development of the avian embryo brain. Among these molecular species, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine accounted for 75-80% of the total PC.

INTRODUCTION

The known influence of phospholipid molecular species on the physical properties of biological membranes and the activity of membrane proteins (1-3) encourages studies on the precise structure of the major phospholipid classes present in order to correlate certain membrane functions with specific lipid compositions. Additionally, systematic studies of developing living systems, which undergo drastic changes in their functional capacities, could be of interest in determining the possible associated lipid compositional changes (4).

Changes in the diet were found to alter the molecular composition of the phosphoglycerides with limited effects on the overall physical properties of the membrane (5,6). In addition, similarities in the molecular species composition of phosphatidylcholine (PC) from homologous tissues of different mammals have been described (7). One is led to believe by these observations that there exists a certain "tissue specificity" in the molecular structure of some major phospholipids (8) which appears to be important in maintaining the functional activity of the tissue.

We describe here the detailed molecular composition of PC from the avian embryonic brain, seeking variation either resulting from development or from differences in avian species.

MATERIALS AND METHODS

Egg incubation, tissue homogenization and lipid extraction were performed as previously described (9). A variable number of embryo brains (50-150 individuals) were pooled according to age.

Isolation of the PC Samples

PCs were obtained directly from the total lipid extract by thin layer chromatography (TLC) on 1.0 mm layers of Silica Gel G (E. Merck A.G., Darmstat, G.F.R.) using chloroform/methanol/water (65:25:4, v/v/v) as the solvent system. Aliquots containing up to 25 mg of total lipids were applied as narrow bands to 20 x 20 cm plates. Bands of PC were detected by spraying the margins of the plates with the Dittmer reagent (10), then scraped off the plates and extracted by successive treatments with 10 vol of chloroform/methanol (2:1, 1:1, 1:2 and 1:9, v/v) and finally methanol. PC isolated by this method appeared as a single spot in 2-dimensional TLC using chloroform/methanol/water (65:25:4, v/v/v) and *n*-butanol/acetic acid/water (60:20:20, v/v/v) as solvent systems.

Conversion of PC into 1,2-Diacyl-3-Acetyl-*sn*-Glycerol

Twenty-five units of phospholipase C (EC 3.1.4.3) from *Clostridium welchii* (Sigma Chemical Co., St. Louis, MO) were dissolved into 8.0 ml of 0.05 M Tris buffer, pH 7.2, and 10.0 ml of 5 mM $CaCl_2$ and washed twice with ethyl-ether to remove lipid contaminants. To this solution were added PC (35 mg) dissolved in freshly distilled ethyl-ether (5.0 ml). The mixture was shaken under N_2 at 29 C for 2 hr, then it was extracted 5 times with ethyl-ether. Combined extracts were evaporated to dryness and weighed. An aliquot containing up to 1 mg of dry extract was analyzed by TLC (0.5 mm thick) using *n*-hexane/ethyl-ether/acetic

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acid (50:50:1, v/v/v) as the solvent system. The 1,2-diacyl-*sn*-glycerol spot was visualized by spraying the plate with a 0.1% Rhodamine-6-G methanol solution or by exposing it to iodine vapor.

The remaining dry extract was acetylated with a 24-fold molar excess of acetic anhydride and 1 vol of anhydrous pyridine at 40 C for 4 min. The reaction was stopped by adding 3.0 ml of anhydrous methanol. Diacyl-glycerol acetates were extracted 5 times with ethyl-ether and the combined extracts were washed with 2 N HCl (2 vol), 2% $NaHCO_3$ (2 vol) and distilled water (3 vol). The organic phase was dried over anhydrous sodium sulfate, concentrated in a rotary evaporator and purified by TLC on 0.5 mm layers of Silica Gel G, using hexane/ethyl-ether/acetic acid (50:50:1, v/v/v) as the solvent system. Bands of diacyl-glycerol acetates were detected with Rhodamine-6-G solution and the 1,2-diacyl,3-acetyl-*sn*-glycerols were scraped off the plate and eluted from the adsorbent with several volumes of chloroform. Initial PC and 1,2-diacyl,3-acetyl-*sn*-glycerols did not show significant differences in their fatty acid compositions (data not shown).

Subfractionation of 1,2-Diacyl,3-Acetyl-*sn*-Glycerol

1,2-Diacyl,3-acetyl-*sn*-glycerols were resolved by argentation ($AgNO_3$-silica gel) TLC on the basis of total number of double bonds per molecule.

Two plates (200 x 200 x 0.5 mm) were prepared as described by Renkonen (11), except that the plates were dried at 120 C for 35 min. The plates were developed using benzene/chloroform/methanol (90:10:1, v/v/v), followed by benzene/chloroform/methanol (90:10:2.5, v/v/v) in order to obtain better resolution of the most highly unsaturated fractions. After development, the plates were sprayed with a 0.05% methanol/water (1:1, v/v) solution of 2',7'-dichlorofluorescein and the different subfractions were located under ultraviolet (UV) light. Those subfractions corresponding to the various degrees of unsaturation were recovered separately by repeated extraction of the gel scrapings with ether. Silver nitrate was eliminated by washing the extracts with 0.1% NaCl solution. The organic extracts were evaporated to dryness in a rotary evaporator after addition of a small amount of methanol to remove the last traces of water. The ratio of the subfractions obtained was determined by gas liquid chromatography (GLC) by adding methyl pentadecanoate (as in internal standard) to the methyl ester derivatives.

Positional Distribution of Fatty Acids in 1,2-Diacyl,3-Acetyl-*sn*-Glycerol Subfractions

The positional distribution of the fatty acids in the 1,2-diacyl,3-acetyl-*sn*-glycerols was determined by hydrolysis with pancreatic lipase (EC 3.1.1.3) from Calbiochem (Los Angeles, CA).

Two to 3 mg of 1,2-diacyl,3-acetyl-*sn*-glycerol acetates were suspended in 1.0 ml of 1 M Tris buffer pH 8.0, 0.25 ml of 2.2% sodium deoxycholate and 0.1 ml of 0.1% $CaCl_2$. The mixture was sonicated for 15 sec in a MSE ultrasonic disintegrator, then 5-6 mg of pancreatic lipase were added and the mixture was incubated for 1 min at 40 C. The reaction was stopped by adding 6 N HCl (3 ml). Free fatty acids and 2-acyl-*sn*-glycerols were recovered by repeated ether extractions and separated by TLC (0.5 mm Silica Gel G layers) with *n*-hexane/ether/acetic acid (50:50:1, v/v/v) as solvent system. Bands were identified using standards, scraped off the plate and directly converted to methyl esters.

Gas Liquid Chromatography

Preparation of the methyl esters and analysis of the fatty acids by GLC were done as described previously (9).

Analytical data represent an average of 2-4 different samples expressed as molar percentages of total fatty acids. Standard deviation for data in Tables I and II and in Figure 1 was no more than 1.9% and 1.0%, respectively, for molar relative abundances higher and lower than 10%.

RESULTS AND DISCUSSION

Fractionation by argentation-TLC of 1,2-diacyl,3-acetyl-*sn*-glycerols derived from PC of chick (13th, 17th and 21st incubation days) and duck embryo brains (17th, 22nd and 28th incubation days) provides (a) the relative abundance (Table I) and fatty acid composition of each fraction, and (b) the positional distribution of acyl groups (GLC data is available from the authors upon request). From these complementary results, the experimental molecular species composition was estimated (Table II).

Five different groups of 1,2-diacyl,3-acetyl-*sn*-glycerol derivatives (Table I) with different degrees of unsaturation were obtained from all the developmental stages considered either in chick or duck embryo brains, i.e., disaturated, mono-, di-, tetra-, and hexaenoic molecules. The relative abundances of all these 1,2-diacyl, 3-acetyl-*sn*-glycerol subfractions remain fairly constant both throughout development and

TABLE I

Molar Relative Abundance of Different Fraction of 1,2-Diacyl,3-Acetyl-*sn*-glycerols Derived from Avian Embryo Brain PC

Molecular classes[a]	Chick (incubation days)			Duck (incubation days)		
	13	12	21	17	22	28
Disaturated	32.1[b]	34.7	29.4	29.4	37.1	35.2
Monoenoic	44.4	46.4	47.7	49.2	46.6	49.0
Dienoic	8.7	8.0	6.6	4.8	4.5	4.0
Tetraenoic	7.9	6.0	7.3	10.6	6.8	7.0
Hexaenoic	6.9	5.0	8.9	5.9	4.9	4.7

[a]Subfractionation of 1,2-diacyl,3-acetyl-*sn*-glycerols is achieved by argentation-TLC on the basis of unsaturation degree (see Methods).

[b]Molar relative abundance of different 1,2-diacyl,3-acetyl-*sn*-glycerol fractions within the whole population from each developmental stage.

TABLE II

Experimental Molecular Species Composition of PC from Developing Avian Embryo Brain[a]

Molecular classes[b]	Chick (incubation days)			Duck (incubation days)		
	13	17	21	17	22	28
16:0/16:0	25.6	30.6	26.8	26.8	32.2	29.1
18:0/16:0	2.6	3.2	1.5	2.6	2.6	4.4
16:1/16:0	3.6	1.8	1.0	1.0	2.0	1.4
18:1/16:0	11.2	13.7	12.6	15.3	19.2	15.4
16:0/18:1[c] (+16:0/16:1)	24.8	27.6	28.9	27.9	22.4	27.3
18:0/16:1 (+18:0/18:1)	2.4	3.4	4.4	4.0	3.8	6.5
16:0/18:2	3.0	2.6	2.2	1.2	1.6	1.2
18:1/18:1 (+18:1/16:1)	2.4	2.6	3.2	3.0	2.6	2.8
16:0/20:4	3.8	3.9	4.0	6.0	3.6	3.9
18:0/20:4	2.0	1.8	2.4	5.4	2.6	2.8
16:0/22:6	3.9	3.9	5.2	3.1	3.4	2.8
18:0/22:6	1.2	1.8	2.2	1.4	1.2	1.2
Total accounted (Σ)[d]	86.50	96.90	94.40	97.70	97.20	98.80

[a]Molecular species with molar relative abundances higher than 1% are represented.

[b]*sn*-1 position/*sn*-2 position.

[c]*Molecular species in brackets indicate minor constituents in not-fully-resolved mixtures.*

[d]Molar percentage of total PC population accounted by the analytical procedure.

between the 2 avian species considered. Nevertheless, the previously reported increase in the absolute amount of PC during the development of the brain tissue (9) provides a steady developmental increase of all molecular species. An overall 1.6 ± 0.4-fold increase can be calculated for each group within the developmental period considered (Fig. 1).

The percentage distribution of individual PC molecular species is shown in Table II. Fifteen major ($\geq$ 2% of total PC population) molecular species are found which do not undergo significant variation in relative abundance either during the course of developmental studies or between the 2 avian species considered. This maintenance of PC composition greatly contrasts with the changes previously described in chick embryo liver (4) and must be partially attributed to the maintenance of the specificity of PC synthesis by the developing brain (Gonzalez-Ros, in preparation).

PC populations are composed of molecules with selective fatty acid distributions, i.e., unsaturated fatty acids are attached primarily to the *sn*-2 position, whereas saturated acyl groups bind mainly to the *sn*-1 glycerol hydroxyl group, as described elsewhere for animal phosphoglycerides (3).

The most abundant molecular species of PC are monoenoic and disaturated, which account for ca. 50 and 30% of the total PC population, respectively. The most abundant disaturate is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine which exhibits increased relative levels on the 17th incubation day in the chick embryo (22nd day in duck) in accordance with previously described increased levels of palmitic acid at the same developmental stage (9). Relative abundance of dipalmitoyl-PC from brain is somewhat higher than in the majority of previously described biological systems (7,12), except for lung tissue, where it exists at an even greater amount (3,7,13).

Another type of disaturated PC at lower levels is 1-stearoyl,2-palmitoyl-*sn*-glycero-3-phosphocholine. There was no detection either of 1-palmitoyl,2-stearoyl or 1,2-distearoyl-*sn*-glycero-3-phosphocholine, which is in agreement with the conclusion reached from studies on liposome permeability where these molecules apparently create a much too rigid and impermeable bilayer structure (14-16). In addition, the absence of molecules such as dilauroyl or dimyristoyl-PC is not surprising because of the observed anomalous behavior of these molecular species in membrane model systems (17).

The monoenoic PC molecular species are present in the developing brain at even higher levels than the disaturated ones and the main components are 1-palmitoyl,2-oleoyl, and 1-oleoyl,2-palmitoyl-*sn*-glycero-3-phosphocholine. 1-Oleoyl, 2-palmitoyl-*sn*-glycero-3-phosphocholine probably is uniquely associated with brain tissue since it cannot be detected in other embryonic organs such as liver or lung (4,13), where it was attributed to a positional selectivity shown by the oleoyl group during the biosynthesis of PC (3,18-20). Alternatively, O'Brien and Geison (21) indicated 1,2-dipalmitoyl and 1-palmitoyl,2-oleoyl PC species as the most characteristic in nerve endings and myelin, respectively.

Other possible combinations of monounsaturated (oleic and palmitoleic) and saturated (palmitic and stearic) fatty acids have also been detected by forming monoenoic molecular species, except that stearic acid was never found attached to the *sn*-2 position.

Dienoic PC molecular species represent ca. 7% of the total PC population. They are formed either by having 2 double bonds in the same apolar chain (1-palmitoyl,2-linoleoyl-*sn*-glycero-3-phosphocholine) or by concomitance of 2 monounsaturated apolar chains within the same PC molecule (1,2-dioleoyl-*sn*-glycero-3-phosphocholine). Both of these have been found previously in the brains of higher animals (3,7).

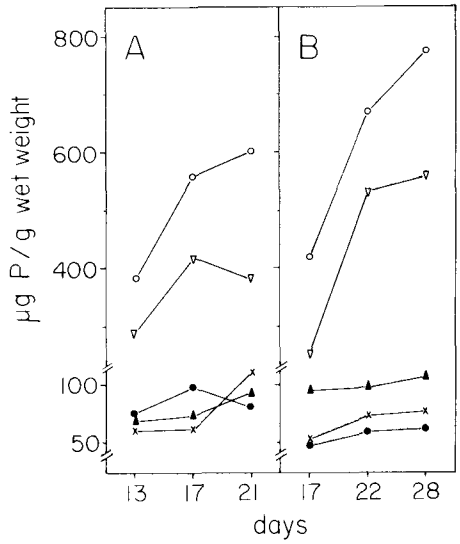

FIG. 1. Developmental changes in the amount of phosphorus contained in molecular species groups of PC with different degrees of unsaturation from chick (A) and duck (B) embryo brain. (▽) disaturated, (○) monoenoic, (●) dienoic, (▲) tetraenoic and (x) hexaenoic molecular species. Phosphorus determinations were performed as previously described (9).

Polyunsaturated PC, composed mainly of tetraenoic and hexaenoic molecular species, have been found with relative abundances between 6-9% in all the developmental stages considered. These are very low values compared to the embryonic chick liver, for example (4). In all cases, preferential combinations of palmitic acid (as different from stearic acid) in the *sn*-1 position with polyunsaturated arachidonic (tetraenoic molecular species) or docosahexenoic (hexaenoic molecular species) acids at the *sn*-2 position are detected. These combinations correspond to polyunsaturated molecular species commonly found in animal tissues, although a predominance of tetraenoic molecular species containing *sn*-1 bound stearic acid, instead of palmitic, also has been reported (3,4,7).

There was no detection of appreciable amounts of dipolyunsaturated (e.g., arachidonic/docosahexenoic, arachidonic/docosahexenoic) molecular species, which agrees with the observed instability of membrane model systems containing high amounts of these phosphoglyceride molecules (15).

Table III shows the molecular composition of PC that would be expected on the basis of a random distribution of the fatty acids present in the initial PC (9). In comparison, experi-

TABLE III

Calculated Molecular Species Composition of PC from the Developing Avian Embryo Brain Assuming Random Distribution of Acyl Groups[a,b]

Molecular classes	Chick (incubation days)			Duck (incubation days)		
	13	17	21	17	22	28
16:0/16:0	29.23	32.97	29.87	26.45	35.29	28.39
18:0/16:0	2.87	2.78	3.66	3.13	3.48	4.63
16:1/16:0	2.76	1.73	1.48	1.87	1.06	0.89
18:1/16:0	13.33	13.39	13.78	14.31	13.66	13.99
16:0/18:1 +16:0/16:1	16.09	15.12	15.26	16.18	14.72	14.88
18:0/16:1 +18:0/18:1	1.57	1.27	1.86	1.91	1.44	2.41
16:0/18:2	1.09	1.30	1.04	0.39	0.30	–
18:1/18:1 +18:1/16:1	7.34	6.14	7.04	8.05	5.70	7.30
16:0/20:4	1.80	2.13	2.07	2.80	2.69	2.74
18:0/20:4	0.17	0.17	0.25	0.33	0.26	0.44
16:0/22:6	1.86	1.90	2.29	1.42	1.77	1.66
18:0/22:6	0.18	0.16	0.28	0.16	0.17	0.27
Total accounted (Σ)	78.29	79.06	78.88	77.00	81.88	79.97

[a]Data on PC fatty acid composition taken from Gonzalez-Ros and Ribera (1979) (9).

[b]Calculation assuming random distribution of acyl groups were done according to Kuksis et al. (1963) (22).

mental values for saturated and monoenoic species containing monounsaturated fatty acids at the *sn*-1 position appear to obey the random distribution, whereas monoenoic species containing monounsaturated fatty acids (mainly oleic acid) at the *sn*-2 position present experimental values deviated positively from the statistical assumption. In the dienoic species fraction, appearance of those pairing saturated and diunsaturated fatty acids apparently is favored whereas the opposite occurs to those containing 2 monounsaturated acyl chains. In addition, it can be observed that experimental values for polyunsaturated species (tetra- and hexaenoic molecules), especially those containing stearic acid, are much higher than those expected from the random distribution assumption. The observed nonrandomness would mean certain acyl groups of PC for the developing brain tend to associate with each other on some basis other than molar concentration. Another possibility is that brain PC represents pooled contributions of molecules from several PC populations, each of which may possess a random distribution for its fatty acids. Whether these results are a reflection of the specificity of endogenous PC synthesis is as yet unknown.

In conclusion, PC from the developing avian brain shows a molecular composition which basically agrees with the trend shown by the brain of higher animals, although it differs from the molecular composition patterns exhibited by other embryonic avian tissues. Monoenoic and disaturated PC species roughly account for 80% of the total PC population where the main components are 1,2-dipalmitoyl, 1-palmitoyl,2-oleoyl and 1-oleoyl,2-palmitoyl-*sn*-glycero-3-phosphocholine. There is a strong maintenance of that pattern both during the embryonic development and between the 2 avian species considered, which may be necessary to keep the functional activity of the embryonic tissue. The mechanism by which this constancy of PC molecular composition is achieved is not understood at this time but may be related to the specificity of endogenous PC synthesis and/or caused by selective transport within the developing encephalon.

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Comparisons of Monoacylglycerols and Diacylglycerols of Varying Fatty Acid Composition As Substrates for the Acylglycerol Kinase(s) of Rat Brain

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ABSTRACT

The properties of diacylglycerol and monoacylglycerol kinase activities present in 90,000 x g pellet and 90,000 x g supernatant fractions from rat brain were examined and compared. Of the properties examined, time course (linear for 10 min), enzyme concentration, pH optimum (7.4-7.5), varying ATP (5 mM) and Mg^{2+} (10 mM) concentrations all showed similar optima for both activities. The optima for acylglycerol (5 mM for diacylglycerols; 3 mM for monoacylglycerols) and deoxycholate concentrations (0.1% for diacylglycerol kinase; 0.03% for monoacylglycerol kinase) differed slightly. Examination of the subcellular distribution for these activities also showed a similar pattern. All fractions showed significant activities, but the most was in the supernatant fraction. The similarities in properties and localization of the 2 kinase activities suggest a single enzyme may function. On this assumption, an extensive study using mono- and diacylglycerols of varying fatty acid composition gave the following results: (a) acylglycerols with the same fatty acid present showed increasing activity in the order: 1-monoacylglycerol, 2-monoacylglycerol and 1,2-diacylglycerols; (b) when saturated fatty acids were present the order of decreasing activity varied directly with increasing chain length for $C_{10} \rightarrow C_{20}$; (c) when one or more unsaturated fatty acids were present good activities resulted, but no clear pattern emerged, although acylglycerols with $_{18:1}$ and $_{18:3}$ fatty acids were more active than those with $_{18:2}$ and $_{20:4}$ fatty acids. These patterns do not support a role for this kinase in producing phosphatidic acids or lyso phosphatidic acids of the correct composition to act as precursors for the de novo synthesis of the predominant 1-stearoyl, 2-arachidonoyl molecular species of phosphatidylinositol.

INTRODUCTION

Michell et al. (1-3), in a number of reviews, has stressed the potential importance of the cycle of phosphatidylinositol (PI) breakdown and resynthesis that may occur in tissues exposed to various stimuli. This cycle involves 4 reactions:

CTP + phosphatidic acid (PA) → CDP-diacylglycerol + PP_i [I]

CDP-diacylglycerol + inositol → PI + CMP [II]

PI → 1,2-diacylglycerol + inositol-1-phosphate (or inositol-cyclic-1,2-phosphate) [III]

1,2-diacylglycerol + ATP → PA + AMP [IV]

Stimuli which affect this cycle appear to act on reaction III. Reactions III and IV clearly show that the formation of 1,2-diacylglycerol (reaction III) and its phosphorylation by ATP to form PA (reaction IV) are important to the cycle. Alternatively, monoacylglycerol may be phosphorylated by a reaction similar to reaction IV to form lyso PA which in turn may undergo acylation to form PA (reactions V and VI).

1-monoacylglycerol or 2-monoacylglycerol + ATP → lyso PA + ADP [V]

lyso PA + acyl CoA → PA + CoASH [VI]

Reactions V and VI permit some modification of the PA pool, depending on the monoacylglycerol participating and the activity of the monoacylglycerol kinase present.

In the past several years, we have studied reactions I and II (4,5) in rat brain preparations using substrates of varying fatty acid composition. The particular aim in these studies was to determine whether the predominant molecular species of PI (i.e., 1-stearoyl, 2-arachidonoyl) that occurs naturally could be formed by either of these reactions, or the 2 reactions acting in concert. The evidence indicates that some but not all of the predominant molecular species observed may occur by reactions I and II. Alternatively, growing evidence indicates a deacylation-reacylation pathway may exist for PI (6-8). Our own studies (6,7) provide evidence for an active deacylation system whereas those of Baker and Thompson (8) have shown that reacylation of 1-acyl-lyso PI readily occurs with high selectivity for arachidonoyl CoA. This cycle or pathway would appear to contribute significantly to the generation of PI species described. A further possibility involves reaction IV and possibly reactions V and VI, particularly if some compartmentalization of the diglyceride from PI breakdown exists which permits recycling of the diglyceride in PI biosynthesis.

It was therefore decided to examine diacylglycerol kinase activity and monoacylglycerol kinase activity in rat brain to evaluate their significance in the steps of PI metabolism described and the generation of the predominant 1-stearoyl, 2-arachidonoyl-species. In the initial experiments done on each enzyme activity, the best optimal conditions for incubation of the enzyme and extraction and measurement of the products formed (^{32}P-PA and lyso ^{32}P-PA) were defined, Using these conditions, diacylglycerols and monoacylglycerols of varying fatty acid composition were then synthesized and compared as substrates. A summary of the findings is described in the following text.

MATERIALS AND METHODS

Materials

The [γ^{32}P] ATP was purchased from the Amersham Corp. (Arlington Heights, IL) as the sodium salt of adenosine 5′-[γ^{32}P] triphosphate (0.5-3.0 mCi/mmol). The following monoacylglycerols: *Rac*-1, 10:0, 12:0, 14:0, 16:0, 18:0, 18:1, 18:2, and 20:4 and *rac*-2, 16:0 and 18:1; 1,2-diacylglycerols: *sn* 1,2-di, 10:0, 12:0, 14:0, 16:0, 18:1; *sn* 1-16:0, 2-18:1, *sn* 1-18:1, 2-16:0, *sn* 1-18:0, 2-18:1, *sn* 1-18:0, 2-18:2; *rac*-1,2 di, 10:0, 16:0, 18:1; and those derived from egg lecithin and pig liver lecithin were purchased from Serdary Research Lab (London, Ont., Canada). The following acyl chlorides, 16:1, 18:2 and 20:4 (arachidonoyl) and acyl glycerols, *rac*-1, 16:1, 18:3 and a *rac* di 20:4 mixture were obtained from Nu-Chek-Prep., Inc. (Elysian, MN). Each acylglycerol was checked for purity and where necessary purified by preparative thin layer chromatography (TLC). The monoacylglycerols were run on Silica Gel H (10-40 μ size)-10% boric acid plates in solvent system I (chloroform/methanol; 98.5:1.5, v/v). The diacylglycerols were run on the same plates in solvent system II (diethyl ether/petroleum ether, 30-60%; 40:60, v/v). Elution of the appropriate area was carried out with chloroform/methanol (60:40, v/v). The additional monoacylglycerols and diacylglycerols used were prepared by chemical synthesis. The 2-monoacylglycerols were prepared as outlined by Mattson and Volpenhein (9). This procedure involved acylation of 1,3-benzylidine glycerol with the appropriate acyl chloride followed by cleavage of the benzylidine group. The crude monoacylglycerols were purified by a 2-stage preparative TLC run using the Silica Gel H plates and solvent systems already described. The plates were first run in solvent system II to migrate the free fatty acid and then after drying in solvent system I in the same direction to separate 1- and 2-monoacylglycerols. The 1,2-diacylglycerols were prepared as described previously by Bishop and Strickland (5). Purification was carried out by preparative TLC on Silica Gel H-10% boric acid plates in solvent system II rather than by runs on silica gel -10% boric acid columns (100-200 mesh).

Tissue and Enzyme Preparations

Male rats of the Sprague-Dawley strain (100-200 g) were used in all experiments. After decapitation, the cerebral hemispheres were rapidly removed and subjected to the homogenation and subcellular isolation procedures described previously (4). Additional washings in ice-cold 0.25 M sucrose were carried out as noted.

Diacylglycerol Kinase Assay

The assay method finally adopted is similar to that described by Lapetina and Hawthorne (10). (The main difference was related to the optimal concentrations of the components added). The basic system for incubation contained in a final vol of 0.5 ml: 1,2-diacylglycerol, 5 mM; sodium deoxycholate, 0.1% (w/v); potassium phosphate buffer, pH 7.4, 50 mM; KF, 10 mM; $MgCl_2$, 10 mM; [γ^{32}P]ATP, 5 mM (specific radioactivity adjusted to ca. 600 cpm/nmol) and enzyme protein 0.1 ml fraction (ca. 0.25 mg protein for microsomal fraction and 0.50 mg protein for supernatant fraction). Appropriate samples (75 μl of 20 mg/ml) of *rac*- or *sn*-1,2-diacylglycerols in hexane were placed in test tubes and the solvent was removed under a stream of N_2. Deoxycholate, phosphate buffer and water were added and the contents (5 mg/ml) of the tube were sonicated for 30 sec. All other components of the system were added (the fluoride last) with the exception of the enzyme preparation, and incubation was carried out at 37 C for 10 min. The enzyme preparation, and incubation was carried out at 37 C for 10 min. The enzyme preparation was then added, the tubes were mixed vigorously on a Vortex (Fischer Scientific Co., Toronto, Canada) for 15 sec and incubation was continued at 37 C for 5 min. At the end of the incubation, the reaction was stopped and each assay tube was extracted and analyzed as described in the next section.

Extraction and Analysis of ^{32}P-incorporation into PA

Four methods of extraction were compared. These included methods described by Prottey and Hawthorne (11), Bremer's group (12),

McMurray (13) and Bishop and Strickland (4). While all methods gave reasonable recoveries, the McMurray method seemed to give a better recovery of counts in the purified lipid extract (e.g., 18,000 cpm vs 14,000-15,000 cpm with the other 3 methods). Because of a consistently higher recovery of counts, the McMurray method was used in subsequent experiments. In this method, the reaction was stopped by the addition of 4.0 ml chloroform/methanol (1:1, v/v). The samples were centrifuged and the extracts were transferred to tubes each containing 2.0 ml chloroform and 0.7 ml of 0.9% NaCl. The phases were separated by centrifugation and the upper phase was discarded. The lower phase was washed 3 times with theoretical upper phase solvent containing NaCl (i.e., chloroform/methanol/0.9% NaCl, 3:48:47, v/v/v). One-half (i.e., 2 ml of the purified lipid extract) was evaporated to dryness and the residue was digested with 1.0 ml of 60% $HClO_4$. The digest was diluted with water to 8.5 ml and the ^{32}P-radioactivity was determined using a Nuclear Chicago scintillation spectrometer. The phosphorus content was determined by the Bartlett method (14) on each of the samples described after counting. The results have been expressed in terms of nanomoles (nmol) [$\gamma^{32}P$]ATP incorporated/mg protein/min. Protein was estimated by the Lowry et al. method (15). Controls (run without added diacylglycerol) were subtracted to give the net incorporation resulting from the diacylglycerol under study.

Monoacylglycerol Kinase Assay

The assay method developed and used was similar to that already described for diacylglycerol kinase. With the exceptions of the monoacylglycerol being 3 mM and deoxycholate being 0.03% the basic system for incubation and the procedure for incubation were identical to the diacylglycerol kinase method.

Extraction and Analysis of ^{32}P-incorporation into Lyso PA

The method described by Bremer's group (12) was found to be the more quantitative for lyso PA recovery and was therefore used for extraction of the ^{32}P-labelled lyso PA formed in the assay described. One-half (0.5 ml of 1.0 ml of butanol extract) of the total extract was analyzed as described for the extract obtained from the diacylglycerol kinase method. The results have been expressed in terms of nmol [$\gamma^{32}P$]ATP incorporated/mg protein/min. Again, as with diacylglycerol kinase activity, appropriate control values were subtracted to give net incorporation resulting from the monoacylglycerol added.

RESULTS

General Properties and Localization of Kinase Enzymes

Initially, a number of experiments were carried out to establish the optimal conditions of incubation for the monoacyl- and diacylglycerol kinases. At the same time, the intracellular distribution of kinase activity was assessed. A close similarity existed between the properties of the 2 kinase activities responsible for the phosphorylation from ATP of monoacyl- and diacylglycerols, with few exceptions. Thus, for each activity, the optimal pH was at 7.4-7.5, the optimal Mg^{2+} concentration was at 10 mM or slightly higher and the optimal ATP concentration was at 5 mM. The system in each case was linear with time for ca. 10 min. Two small differences noted were in the optima for substrate concentrations (3 mM for monoacylglycerols vs 5 mM for diacylglycerols) and for deoxycholate (0.3% for monoacylglycerol kinase activity vs 0.1% for diacylglycerol kinase activity). Most of these conditions agree with those described by Lapetina and Hawthorne (10) for diacylglycerol kinase in rat cerebral cortex.

The localization of both kinase activities in the different cellular components derived from rat brain by differential centrifugation showed another close similarity in the pattern of the activities (Table I). While activity appeared in all fractions, most of it was in the supernatant fraction derived from centrifugation at 90,000 x g for 30 min for both kinase activities. The unwashed microsomal pellet (i.e., 90,000 x g) was relatively high in activity, but 2 washings with sucrose buffer reduced the activity to an average of less than 10% of that in the supernate or total homogenate. This indicates only a "loose" or nonintegral association of these kinase activities with the membranous components contributing to the 90,000 x g pellet.

Comparisons Among Monoacylglycerol and Diacylglycerol Substrates of Varying Fatty Acid Composition

In all the experiments carried out in this study, the optimal conditions already discussed were used with the different substrates, cofactors and deoxycholate. Tables II and III summarize the data obtained for both diacylglycerols and monoacylglycerols of varying fatty acid compositions using a microsomal and a supernatant fraction from rat brain. With few exceptions, the kinase activities were higher and

TABLE I

Intracellular Distribution of Diacylglycerol and Monoacylglycerol Kinases in Rat Brain

Tissue fraction[a]	[γ-^{32}P]-ATP incorporated per min into lipid[b]			
	nmol/g wet wt		nmol/mg protein	
I. Diacylglycerol kinase (1,2-dioleoyl-*rac*-glycerol, 5 mM, used as substrate)				
A. (Expts 1 and 2)	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Homogenate	563	636	10.6	9.6
1,000 x g pellet[c]	363	227	25.1	8.9
12,000 x g pellet[c]	252	223	7.7	7.1
90,000 x g pellet[c]	5	69	2.2	6.5
90,000 x g supernate	586	672	28.6	29.2
B. (Expts 3 and 4)	Expt. 3	Expt. 4	Expt. 3	Expt. 4
Homogenate	663	752	11.6	7.5
90,000 x g pellet (unwashed)	306	197	15.3	9.2
Wash No. 1	67	22	22.2	9.0
Wash No. 2	16	4	5.2	1.5
90,000 x g pellet[c]	95	44	6.3	5.0
90,000 x g supernate	551	842	20.4	26.3
II. Monoacylglycerol kinase (2-oleoyl-*rac*-glycerol, 3 mM, used as substrate)				
(Expts 5 and 6)	Expt. 5	Expt. 6	Expt. 5	Expt. 6
Homogenate	93.6	75.6	0.94	0.74
1,000 x g pellet[c]	12.2	17.8	0.47	1.19
12,000 x g pellet[c]	25.8	20.2	0.71	0.57
90,000 x g pellet[c]	6.18	4.72	0.59	0.59
90,000 x g supernate[c]	124	119	4.00	4.17

[a]Cell fractionation was carried out as described in Materials and Methods.

[b]Diacylglycerol and monoacylglycerol kinase activities were measured under the optimal conditions described in Materials and Methods. Activities are expressed as given in the subheadings.

[c]Pellet washed twice in ice-cold 0.25 M sucrose.

usually more stable in the supernatant fraction for all of the monoacyl- and diacylglycerols studied. Where comparisons can be made in Table II, the *sn*-forms of the diacylglycerols usually show higher activities than the corresponding *rac*-forms (range 91-155% for *sn*-form/*rac*-form x 100). The monoacylglycerol kinase activity (using *rac*-2-monoolein) was about 40% of the diacylglycerol kinase activity (using *rac*-1,2-diolein). The 2-monoacylglycerols (Table III) showed higher activities than the corresponding 1-monoacylglycerols.

Examination of the results shown in Table II for the 1,2-diacylglycerols indicates a number of patterns. The diacylglycerols with 2 saturated fatty acids of the same composition showed greater activity for shorter chain (C_{10} or C_{12}) fatty acids than for longer chain ($C_{14} \rightarrow C_{18}$) fatty acids. This order of activity is probably directly related to the physical ability to disperse these diacylglycerols despite good, uniform dispersions being obtained after sonication. Once one or more unsaturated fatty acids are introduced into the diacylglycerol, a considerable increase in kinase activity is evident in both the *sn*- and *rac*-series. It makes little difference whether the unsaturated fatty acid (e.g., oleic, linolenic or arachidonic) is at the 1- or 2-positions. All of the *sn*-diacylglycerols with one or more unsaturated fatty acids showed activities almost as high or higher than the activity observed for *rac*-1,2-diolein. With the *rac*-diacylglycerols, those with one or more unsaturated fatty acids always showed higher activities than the corresponding diacylglycerols with saturated fatty acids, but in most instances the activities (15-105% of *rac*-1,2-diolein) were less than the activity observed for *rac*-1,2-diolein. The diacylglycerols containing $C_{20:4}$ fatty acid alone or with $C_{18:0}$ stearic acid do show appreciable activity (15-31% of *rac*-1,2-diolein), but no selectivity in their favor is evident. This selectivity would be required for the kinase to play a significant role in the de novo generation of a PA with the correct composition to form the predominant 1-stearoyl-2-arachidonoyl species of PI.

The data shown in Table III for *rac*-1- and *rac*-2-monoacylglycerols also show certain patterns appart from the higher activity already

TABLE II

Comparison of Diacylglycerols of Varying Fatty Acid Composition as Substrates for Phosphatidic Acid Formation by Microsomal and Supernatant Fractions of Rat Brain

Diacylglycerol (5 mM)	Microsomal fraction		Supernatant fraction	
	Specific activity[a]	Relative activity[b]	Specific activity[a]	Relative activity[b]
1,2-dioleoyl-*rac*-glycerol	8.64 (22)	1.00	11.5 (22)	1.00
sn-forms				
1,2-dicaproyl-	5.66 (2)	0.66	7.46 (2)	0.63
1,2-dilauroyl-	6.88 (2)	0.78	8.28 (2)	0.70
1,2-dimyristoyl-	1.91 (2)	0.22	3.86 (2)	0.33
1,2-dipalmitoyl-	1.55 (3)	0.18	3.74 (3)	0.32
1-palmitoyl,2-oleoyl-	14.3 (2)	1.67	16.6 (2)	1.43
1-stearoyl,2-oleoyl-	12.1 (4)	1.40	14.2 (2)	1.22
1-stearoyl,2-linoleoyl-	7.18 (3)	0.83	10.4 (3)	0.89
1,2-dioleoyl-	13.4 (3)	1.55	14.0 (3)	1.20
1-oleoyl,2-palmitoyl-	12.4 (2)	1.42	16.1 (2)	1.38
From egg lecithin	7.84 (4)	0.90	5.00 (3)	0.43
From pig liver lecithin	8.28 (2)	0.96	12.5 (6)	1.07
rac forms				
1,2-dicaproyl	5.18 (2)	0.60	5.94 (2)	0.51
1,2-dipalmitoyl-	1.05 (3)	0.12	4.06 (3)	0.35
1,2-distearoyl-	0.51 (3)	0.06	1.05 (2)	0.10
1-stearoyl,2-oleoyl-	2.80 (4)	0.32	4.02 (4)	0.35
1,2-dioleoyl-	as above	1.00	as above	1.00
1-oleoyl,2-stearoyl-	1.56 (4)	0.18	2.20 (4)	0.19
1-stearoyl,2-linolenoyl-	9.14 (4)	1.05	10.7 (4)	0.92
1-linoleoyl,2-stearoyl-	7.34 (4)	0.85	9.02 (4)	0.78
1-stearoyl,2-arachidonoyl-	1.85 (2)	0.22	1.77 (2)	0.15
1-arachidonoyl,2-stearoyl-	2.70 (2)	0.31	2.16 (2)	0.19
1,2-diarachidonoyl	2.24 (2)	0.26	2.28 (2)	0.20

[a]The specific activity is expressed as nmol [γ^{32}P]ATP incorporated/mg protein/min. Number of observations are in parentheses.

[b]The relative activity denotes the activity relative to 1,2-dioleoyl-*rac*-glycerol set at 1.00.

noted for the 2-monoacylglycerols. The 1- and 2-monoacylglycerols with saturated fatty acids show a decreasing activity as chain length is increased (e.g., $C_{10} \rightarrow C_{20}$) and also much less activity than corresponding monoacylglycerols with unsaturated fatty acids. The *rac*-2-monoacylglycerols with unsaturated fatty acids (palmitoleic, oleic, linoleic and arachidonic) all showed comparable activities (86-106% of 2-monoolein). With one exception (monolinolein), the same pattern was noted for the *rac*-1-monoacylglycerols with unsaturated fatty acids.

DISCUSSION

In this study, the properties exhibited by diacylglycerol kinase activity toward 1,2-dioleoylglycerol and by monoacylglycerol kinase activity toward 2-oleoylglycerol in rat brain preparations was first examined and compared. As already noted, the properties (time course; enzyme concentration, diacylglycerol, ATP and Mg^{2+}-concentrations; activation by deoxycholate and pH optimum) of the diacylglycerol kinase observed in both a 90,000 x g pellet (microsomal) and a supernatant fraction from rat brain were almost identical to those reported by Lapetina and Hawthorne (10). Apparently, no comparable study has been reported on monoacylglycerol kinase since the first report by Pieringer and Hokin (16) on a partially purified preparation obtained from a deoxycholate extract of either cytoplasmic particulate or microsomal fraction from guinea pig or calf brain. Although a careful identification of product (i.e., as lyso PA) is made, optimal conditions for substrates and other factors are not noted. In most instances, the concentrations were lower than the optima observed in this study. The very close similarity between the properties exhibited by the above 2 kinase activities would be consistent with the view that a single kinase

TABLE III

Comparison of Monoacylglycerols of Varying Fatty Acid Composition as Substrates for Lysophosphatidic Acid Formation by Microsomal and Supernatant Fractions of Rat Brain

rac-Monoacylglycerols (3 mM)	Microsomal fraction		Supernatant fraction	
	Specific activity[a]	Relative activity	Specific activity[a]	Relative activity
2-oleoyl-	3.40 (15)	1.00[b] ...	4.82 (15)	1.00[b] ...
1-oleoyl-	0.95 (2)	0.28 1.00[b]	1.86 (2)	0.39 1.00[b]
2-acylglycerols:				
2-palmitoyl	0.27 (2)	0.08 ...	1.57 (2)	0.33 ...
2-stearoyl	0.67 (4)	0.20 ...	1.49 (4)	0.33 ...
2-arachidoyl	0.55 (3)	0.16 ...	0.89 (3)	0.19 ...
2-palmitoleoyl	3.60 (2)	1.06 ...	4.14 (2)	0.86 ...
2-oleoyl	as above	1.00 ...	as above	1.00 ...
2-linoleoyl	3.94 (5)	1.16 ...	5.44 (5)	1.13 ...
2-arachidonoyl	3.84 (4)	1.02 ...	4.90 (4)	1.02 ...
1-monoacylglycerols:				
1-caproyl	0.31 (2)	0.09 0.33	0.83 (2)	0.17 0.44
1-lauroyl	0.32 (2)	0.09 0.33	0.81 (2)	0.17 0.44
1-myristoyl	0.19 (3)	0.06 0.20	0.68 (3)	0.14 0.37
1-palmitoyl	0.10 (2)	0.03 0.11	0.34 (2)	0.07 0.18
1-stearoyl	0.20 (4)	0.06 0.21	0.44 (4)	0.09 0.24
1-arachidoyl	0.10 (3)	0.03 0.11	0.10 (3)	0.02 0.06
1-palmitoleoyl	0.97 (2)	0.29 1.02	1.36 (2)	0.28 0.73
1-oleoyl	as above	0.28 1.00	as above	0.39 1.00
1-linoleoyl	0.57 (3)	0.17 0.59	1.09 (3)	0.23 0.58
1-linolenoyl	1.22 (2)	0.36 1.28	1.42 (2)	0.29 0.76
1-arachidonoyl	0.79 (5)	0.23 0.82	1.35 (5)	0.28 0.72

[a]The specific activity is expressed as nmol [γ-^{32}P]ATP incorporated per mg protein/min. Numbers of observations are in parentheses.

[b]All activities are expressed relative to 2-oleoyl-*rac*-glycerol set at 1.00 in first column. In the second column, activities of the 1-monoacylglycerols are expressed relative to 1-oleoyl-*rac*-glycerol set at 1.00.

activity is responsible for the phosphorylation by ATP of mono- and diacylglycerols. Further investigation with purified preparations of acylglycerol kinase(s) is necessary to establish this possibility unequivocally.

The experiments under study on the intracellular localization of the 2 kinase activities show several findings. Some activation of the activities is evident in the subcellular fractions since total recoveries are 1.5-2 times that of the total homogenate. Possibly this results from a differential activation effect of the deoxycholate present in the assay system. Each subcellular fraction after 2 washings has significant activity, although that of the 90,000 x g pellet is low, indicating a wide intracellular distribution of kinase activity. This is not inconsistent with findings from other laboratories (10,13) for diacylglycerol kinase. The relative ease of removal of the kinase activities from the 90,000 x g pellet suggests the enzyme(s) responsible for these activities are "nonintegral or extrinsic" in their location in the membrane of this fraction. In fact, the relatively large amount of enzyme activity in the supernatant fraction might reflect a pattern of localization in which the acylglycerol kinase activities are loosely bound on the membrane and are released during homogenation. Again, the close similarity in pattern of intracellular localization would support the view that a single kinase enzyme is responsible for the phosphorylation of mono and diacylglycerols.

On the assumption that a single enzyme is responsible for the activities described and on the basis of the experiments reported in Tables II and III in which comparisons of acylglycerols of varying fatty acid composition are noted, a number of conclusions can be made. A comparison of the acylglycerols of comparable fatty acid composition (e.g., $_{18:1}$ oleic) shows the following order of activity: diacylglycerol > 2-monoacylglycerol > 1-monoacylglycerol. When saturated fatty acids are present in all 3 types of substrate, the activity is lower than when one or more unsaturated fatty acids are

present. Also, as the chain length of the saturated fatty acids is increased from $C_{10} \rightarrow C_{20}$ there is a decrease in the activity of the acylglycerol as a substrate for the kinase enzyme. No pattern of selectivity or specificity is evident among unsaturated fatty acids substituted in the acylglycerols. Relatively good activities are obtained when on or more unsaturated fatty acids are present. The highest activities were obtained with $_{18:1}$ and $_{18:3}$ fatty acids whereas the lowest were obtained with $_{18:2}$ and $_{20:4}$ fatty acids.

This study with acylglycerols of varying fatty acid composition clearly indicates that no pattern of selectivity or specificity is evident which favors the generation of PAs or lyso PAs with a predominance of the 1-stearoyl-2-arachidonoyl molecular species which, as has been noted earlier, would be necessary if the kinase were to play a significant role in the generation of the molecular species of PI described. A similar conclusion has been reached by Holub and Piekarski (17) using 1-saturated (palmitic or stearic), 2-unsaturated monoenoic to hexaenoic (oleic, linoleic, arachidonic and docosahexenoic) substituted 1,2-diacylglycerols in a rat brain microsome system. Neither study, however, excludes the possibility of the acylglycerol kinase, in particular that activity toward 1,2-diacylglycerols, from functioning in either a compartmentalized or noncompartmentalized situation on a diacylglycerol pool under physiological conditions to regenerate PA or the correct molecular species for PI biosynthesis.

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Separation and Identification of Prostaglandin A_1 in Onion[1]

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ABSTRACT

The separation of a fraction corresponding to prostaglandin A_1 from yellow onion *(Allium cepa)* and subsequent purification of that fraction as prostaglandin A_1 has led to the identification of prostaglandins in a plant material for the first time. Kilogram quantities of onions were processed and purified by extraction procedures, column chromatography and thin layer chromatography (TLC). Prostaglandin A_1 was characterized and identified by a combination of comparative TLC, gas chromatography-mass spectrographic analysis and blood pressure lowering properties. The results of these experiments are consistent with standard prostaglandin A_1. It is concluded that prostaglandin A_1 is present in onion.

INTRODUCTION

Our previous report (1) indicated the possible presence of prostaglandin A_1 (15-hydroxy-9-ketoprosta-10,13-dienoic acid) in yellow onions *(Allium cepa)*. In that report, a limited thin layer chromatographic study, infrared spectral analysis, evidence of prostaglandin A_1 (PGA_1) being converted to PGB_1 upon base treatment and evidence that the onion extract corresponding to PGA_1 lowered the blood pressure in rats were presented. However, the unequivocal presence of prostaglandins in plants has not been reported to date.

Albro and Fishbein (2) in 1971 reported the results of an attempt to isolate prostaglandins from wheat bran. Also, in 1979, Panosyan et al. (3) isolated compounds from *Byronia alba* which had prostaglandin-like activity. In both studies, these compounds were long chain hydroxy unsaturated fatty acids which behaved similarly to one of the prostaglandins. Likewise, another study (4) has indicated the isolation of prostaglandin-like compounds from *Saccharum officinarum*, *Musa paradisiaca* and *Cocos nucifera lin.*

Two recent investigations (5,6) have given some experimental evidence that prostaglandins may exist in plants. Bild et al. (5) isolated an isoenzyme from soybean and demonstrated that this enzyme was capable of converting arachidonic acid to a prostaglandin, showing certain plants do have the enzymatic capability to make the conversion of free fatty acids to prostaglandins. Groenewald and Visser (6) have shown that applied prostaglandins to the aseptic excised shoot apices of *Pharbitis nil* hastens the time of flowering. They present a hypothetical scheme for the possible regulation of flowering by prostaglandins and by prostaglandin antagonists (some phenolic acids).

In the initial stages of our study, numerous plants were screened, and it was found that several plants had components which corresponded to prostaglandins on thin layer chromatographic (TLC) separations. Of those screened, onion was the most promising. In this report, we show that prostaglandin A_1 is present in onion by comparing the mass spectra (MS) of the onion component to that of authentic PGA_1 samples.

EXPERIMENTAL PROCEDURES

Materials

Prostaglandin standards were provided by the Upjohn Co. Reagents for this study were ACS analytical grade or spectral grade. The eluants for the column chromatography were usually distilled prior to use.

Separation of the Crude Onion Fraction

The method for the separation and subsequent purification of the lipid-soluble fatty acid fraction from yellow onion which corresponds to the PGA fraction was the procedure used in our previous work (1). This procedure was the method used by Lee and coworkers (7) for the isolation and identification of PGA_2 from rabbit medulla. Since the procedure is published elsewhere, the analysis is summarized as follows: ca. 200 g of yellow onions were homogenized for 3 min with 200 ml of 5 mM sodium phosphate buffer. This mixture was filtered and ethanol added to make the solution 80% in ethanol (v/v) to precipitate the protein. This ethanol solution was placed in a 3 C

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environment for 15-20 hr. After this period, the solution was filtered and the ethanol removed with a rotary vacuum evaporator at 35 C. The pH of the remaining mixture was adjusted to 8 with 5 N sodium hydroxide and partitioned against equal volumes of petroleum ether 3 times; the ether layer was then discarded. The pH of the solution was adjusted to 3 with 2 N citric acid, partitioned against equal volumes of petroleum ether 3 times and the ether layer was discarded. The solution was then extracted with equal volumes of chloroform 3 times, and the chloroform layers were pooled. The chloroform solution was then evaporated at 35 C. The residue was removed from the vessel by washing with 3 ml of ethanol/chloroform (1:1, v/v) 3 times. The solution was then evaporated in a water bath by passing a stream of N_2 over the sample. This fraction was designated as the crude onion extract.

Purification of the Crude Onion Extract by Column Chromatography

Sil-A-200 silicic acid prepared for resolution of acidic mixtures with a mesh size 60-200 was obtained from Sigma Chemical Company (St. Louis, MO). Spectrograde methanol, benzene and ethyl acetate were used without further purification. The silicic acid column chromatographic separation procedure is similar to that previously reported (8,9).

Silicic acid (7.89 g) was combined with 25 ml of the eluting solvent benzene/ethyl acetate (9:1, v/v). This slurry was poured into a column 1 cm in diameter and 50 cm length. The final silicic acid column height was 21.5 cm. A crude onion extract sample weighing ca. 70-100 mg was dissolved in the benzene/ethyl acetate (9:1) and introduced on the column head. The elution rate was adjusted to ca. 1 ml/min, and a 500 ml fraction of the benzene/ethyl acetate (9:1, v/v) was collected. The eluting solvent was changed to benzene/ethyl acetate (8:2, v/v), and a 500 ml fraction was collected. This fraction, which corresponds to the PGA_1, was taken to dryness with a rotary vacuum evaporator at 35 C. The residue in the flask was removed by washing the vessel with 3 ml of ethanol/chloroform (1:1) 3 times. The sample was then taken to dryness in a water bath by passing N_2 over the solution.

TLC Studies

Silicic AR TLC-7GF Chromatographic Sorbent was obtained from Mallinckrodt. Analytical reagent grade chloroform, methanol, acetic acid, ethyl acetate, formic acid, dioxane, benzene, tetrahydrofuran (THF) and methylene dichloride were used without further purification.

Further purification of the PGA_1 fraction collected from the column chromatography was performed by TLC using chloroform/THF/acetic acid (10:2:1) as the solvent. The spot corresponding to PGA_1 was removed from the sorbent by adding chloroform/methanol (1:1) to the sorbent and filtering. The solution was then evaporated to dryness with a rotary vacuum evaporator at 35 C and the residue taken up in 0.5 ml of methanol. To this solution, 5 ml of water was added and the pH was adjusted to 3 with 2 N citric acid. This was extracted with equal volumes of chloroform 4 times. The chloroform extracts were pooled and washed with water until the pH of the water was ca. pH 7. The chloroform solution was then evaporated to dryness with a rotary vacuum evaporator at 35 C. The residue was removed from the vessel by washing with 3 ml of chloroform/ethanol (1:1) 3 times. This solution was then evaporated in a water bath by passing a stream of N_2 over the sample. This fraction was submitted to further TLC studies which included 10 different solvent systems. (The procedure just described was also used to prepare samples for the gas chromatography-mass spectrometry (GC-MS) studies and the blood pressure lowering studies.)

In the TLC study, ca. 50-100 μg of the onion component and 50 μg PGA_1 were spotted on the activated plates. The solvent front was allowed to migrate up to the plate for a distance of 10 cm. The plates were air dried and were visualized by short and long ultraviolet (UV) light. Permanent thin layer chromatograms were developed by spraying with a 10% phosphomolybdic acid in ethanol and heating at 100 C until visible.

GC-MS Analysis of Onion Extract

A portion of the previously described onion extract was further purified by TLC on Silica Gel G(F) sorbent using chloroform/methanol/water (15:1:0·03). This TLC study was done independently from the previous TLC studies. The compounds were chromatographed on 2.5 cm x 7.5 cm plates and visualized with UV light or by spraying with sulfuric acid/ethanol (1:1) followed by heating to 100 C. For preparative procedures for GC-MS analyses, compounds were chromatographed on 20 cm x 20 cm TLC plates, visualized with UV light and recovered by treating the plate with "Strip-Mix" (Applied Science). Areas containing prostaglandin A_1 were removed and eluted with chloroform-ethanol (1:1).

Prostaglandin methyl esters (MEs) were prepared by reaction with excess diazomethane

in ether for 2 min at room temperature.

Trimethylsilyl ether (TMS) and trimethylsilyl estertrimethylsilyl ether (*bis*-TMS) derivatives were prepared by reaction of the prostaglandin-MEs and free prostaglandins, respectively, with 50 μl of N,O-*bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) (Pierce Chemical Co.) at 70 C for 1 hr. The samples were evaporated under N_2 and dissolved in 20 μl of dry cyclohexane. A few crystals of anhydrous Na_2SO_4 were added to the vials to maintain dryness.

GC-MS was carried out on a Finnigan 1015D GC-MS interfaced with a Systems Industries System 150 data acquisition and control system. The compounds were chromatographed on a 1.5 m x 2 mm id glass column of 1% OV-17 on 80/100 mesh Gas Chrom Q (Applied Science, State College, PA). The column temperature was maintained at 200 C for chromatography of ME-TMS derivatives and at 250 C for *bis*-TMS derivatives, the injector temperature was 240 C, interface was 250 C and the He carrier gas flow was 20 ml/min.

Blood Pressure Studies

Male Wistar rats were anesthetized with nembutal sodium at a dosage of 5 mg/100 g wt. An I.P.A. No. 23-G x 3/4 infusion set was introduced into the femoral vein to give bolus injections of authentic PGA_1 and the onion extract corresponding to PGA_1. The carotid artery of the animal was connected with a No. PE 90 polyethylene tube which was connected to a Statham transducer for blood pressure measurement using a Grass Polygraph.

RESULTS

The TLC comparison of the onion component to that of standard PGA_1 is given in Table I. The study included 10 solvent systems. Notations of the solvent systems are generally those reported by Andersen (10). Most of the solvent system TLC separations were done in duplicate. The spots were identified either by UV or by phosphomolybdic acid development. In all trials, the R_f values reported for the component in onion agreed well with that of the standard PGA_1.

One sample representing 22 kg of onion was further purified using the $CHCl_3$-MeOH-H_2O TLC system; it was then converted to the methyl ester trimethylsilyl derivative for GC-MS analysis. The onion extract contained a component which had a retention time of 6.52 min which was identical to the reference PGA_1-ME-TMS. Except for the absence of the molecular ion, the mass spectrum of the onion component corresponding to PGA_1-ME-TMS was identical to the reference derivative.

Allowing for a lower relative intensity of the high mass ions observed in the spectra obtained using a quadrupole mass spectrometer, the spectra of the onion compound and reference PGA_1-ME-TMS were comparable to the published spectrum of PGA_1-ME-TMS by Sweetman et al. (11).

Another sample representing 15 kg of onion was prepared; the methyl ester trimethylsilyl derivative was made and analyzed by GC-MS. The column for this separation was 1% SP 2250 operated at 240 C with a helium gas flow of 30 ml/min. A component with a retention time of 3.93 min was observed which was identical to the retention time of the reference PGA_1-ME-TMS. The onion component whose retention time was 3.93 min included a molecular ion at m/e = 422 which is the same as that of the reference PGA_1-ME-TMS. A comparison of the 2 peaks is given in Figure 1. Characteristic fragment ions included M+ = 422, m/e 351 (M - 71), m/e 323 (M - 99), m/e 319 (M - 71 - 32), m/e 301 (M+ - 31 - 90), m/e 261 (M+ - 71 - 90), m/e 247, m/e 229, m/e 199 and m/e 173 (11-13).

GC-MS analysis of the onion extract as the trimethylsilyl ester-trimethylsilyl ether (*bis*-TMS) derivative revealed a compound with a retention time on OV-17 (2.12 min) indistinguishable from that of the reference PGA_1-*bis*-TMS (2.09 min). The mass spectrum of the onion unknown was similar to the spectrum of the reference PGA_1-*bis*-TMS with characteristic fragment ions at M+ = 480, m/e 465 (M - 15) m/e 409 (M - 71), 381 (M - 99) and 319 (M - 71 - 90).

Figure 2 shows the blood pressure depression profile of 10 μg standard PGA_1 with that of 1000 μg of the onion extract corresponding to PGA_1. With authentic PGA_1, the blood pressure fell from 120/90 to 60/25 mm Hg returning to the baseline over a period of 10-15 min, a response which is typical for this compound. With the purified onion extract, the blood pressure fell from 125/100 to 85/45 mm Hg, again, returning to the baseline in 10-15 min. The characteristics of the depressed blood pressure with the onion extract closely resembled that of authentic PGA_1.

Responses of 200 μg of onion extract were also noted. The reduction of blood pressure was not as well defined. The blood pressure response observed for 10 μg of authentic PGA_1 was quite similar to the 1000 μg sample of onion extract. Semiquantitatively evaluating the response in terms of the quantity of active material in the onion extract, it can be estab-

TABLE I

Thin Layer Chromatography Studies of PGA Fraction in Onions

Solvent system notation	Solvent components	Concentration (volume ratios)	R_f values for PGA_1	R_f values for onion extract
C-I	Chloroform-THF-acetic acid	10:2:1	0.83 0.84	0.83 0.84
H-I	Hexane-methylene dichloride-THF-acetic acid	6:2:2:1	0.67 0.67	0.67 0.67
H-II	Hexane-methylene dichloride-THF-acetic acid	30:10:3:3	0.78 0.75	0.77 0.76
D-II	Benzene-dioxane-acetic acid	40:10:1	0.69 0.52 0.52	0.69 0.52 0.54
D-III	Benzene-dioxane-acetic acid	40:20:2	0.60 0.52	0.59 0.53
D-IV	Benzene-dioxane-acetic acid	40:40:2	0.60 0.61	0.62 0.62
F-II	Ethyl acetate-formic acid	200:1	0.29 0.30	0.30 0.29
F-III	Ethyl acetate-formic acid	400:1	0.33 0.34	0.34 0.35
O-I	Chloroform-methanol-acetic acid	18:2:1	0.87 0.78	0.87 0.78
O-II	Chloroform-methanol-water	15:1:0.03	0.26	0.26

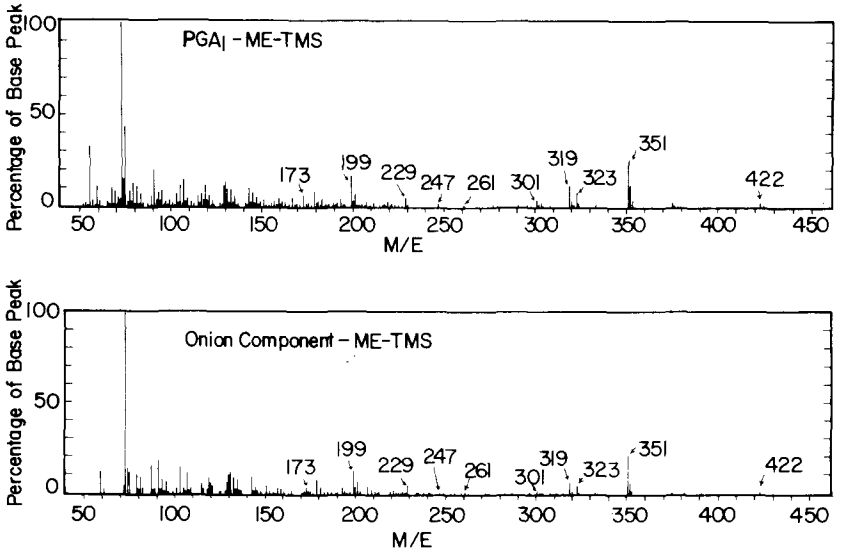

FIG. 1. Mass spectra of PGA_1-ME-TMS (t_r = 3.93 min) and the onion component ME-TMS (t_r = 3.93 min) eluted from the 1% SP 2250 gas chromatography column.

lished that it was ca. 1 part in 100 of the extract, or ca. 1 part per million (ppm) in onion, knowing the initial onion sample size.

GC-MS studies of this investigation indicate approximately the same order of magnitude for the concentration of PGA_1 in onion. It was assumed that the procedure that was used for the isolation and purification of the prostaglandin fraction would yield a relatively pure sample. It became evident from the GC-MS work that the sample was not pure PGA_1, but a mixture of several components. In that mixture, PGA_1 was identified by the GC-MS portion of this investigation.

DISCUSSION

This study demonstrates that PGA_1 is present in onion. The previous report (1) of certain chemical and structural similarities of the component in onion corresponding to PGA_1 and the evidence produced here helps confirm the presence of a prostaglandin in plant material.

Since prostaglandins have, up to this time, been exclusively found in animal species, this study represents an interesting appendix to the investigations of Bild et al. (5) and Groenewald and Visser (6). As previously mentioned, it was demonstrated (5) that lipoxygenase-2 isolated from soybeans was capable of catalyzing the oxygenation of arachidonic acid to form significant amounts of prostaglandin product. Since it is now established that plant materials

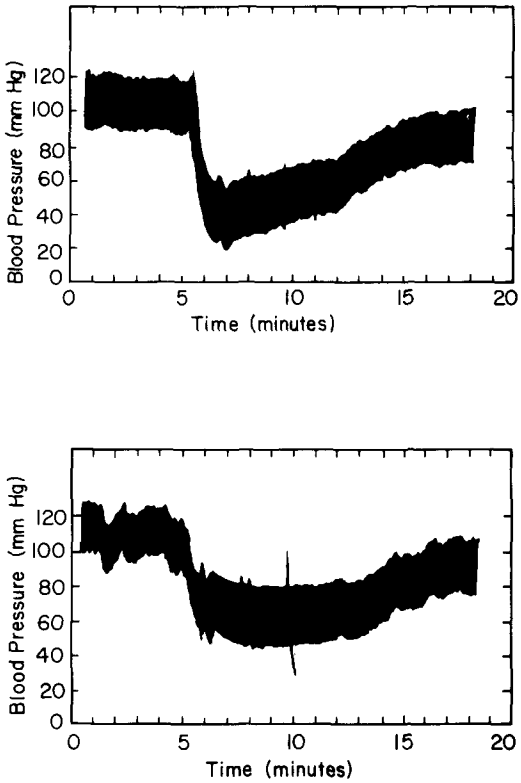

FIG. 2. Effect of the intravenous injection of 10 μg PGA_1 in 0.5 ml buffer (top) and 1,000 μg of onion extract corresponding to PGA_1 in 0.5 ml buffer (bottom).

do contain the enzymatic capability to convert the precursor free fatty acids to prostaglandin, it is reasonable to speculate that prostaglandins do exist in plant material. Although it is beyond the scope of this study to speculate on the function of prostaglandins in onion, the report of Groenewald and Visser suggests quite strongly that prostaglandins may play a role in the flowering of *P. nil* plantlets (excised shoot apices). The results of the experiments imply (a) the presence of prostaglandins in plants, and (b) that the role of these prostaglandins is directly related to flowering, which is inhibited by certain phenolic acids (gentistic acid, acetylsalicylic acid and salicylic acid).

Our first studies used ca. 1 kg samples of whole onion for the preparation of the onion fraction corresponding to PGA_1. The sample sizes were increased to 15 and 22.5 kg and it was from these 2 samples that the fraction corresponding to PGA_1 produced the confirming MS identification as PGA_1. Although the data presented supports the conclusion that the product is a prostaglandin of the A type, A_1 in particular, it is possible there is a conversion of PGE_1 to PGA_1 by dehydration in the cyclopentyl ring, caused by the analytical procedures of separation and purification. We have not tested this hypothesis, but we do feel that evidence of a prostaglandin (whether of type A or E) in plant material is established on the basis of our results.

ACKNOWLEDGMENTS

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Autoxidation of Methyl Esters of Cyclopentenyl Fatty Acids

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ABSTRACT

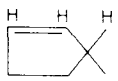
The early stages of the autoxidation of methyl hydnocarpate, chaulmoograte and gorlate in air have been examined at 40, 60 and 80 C, and the initial products have been compared by several methods with those derived from methyl oleate and linoleate autoxidized at 60 C. To supplement information about oxygen absorption and peroxide development in relation to time, other information about the early products, and some information about the reduced products, have been obtained by ultraviolet (UV) and infrared (IR) spectrophotometry, and by thin layer chromatography (TLC). The kinetic and other data presented in this study strongly support the conclusion that the methyl esters of cyclopentenyl fatty acids yield initial autoxidation products that, although they are primarily peroxides, differ in some ways (as expected) in the kinetics of their formation and their chemical nature, compared to those of oleate and linoleate. Nevertheless, all the data obtained strongly support the surmise that the peroxides are formed autocatalytically by a chain mechanism, and that secondary products not derived from peroxide decomposition, are formed pari passu in lesser, but increasing amounts with increasing temperature, probably from free radical intermediates. The autoxidation of esters of cyclopentenyl fatty acids has potential importance in several ways, 3 of which are mentioned briefly.

INTRODUCTION

It has been known for hundreds of years that chaulmoogra oils, which are found mainly in the seeds of shrubs and trees of the family Flacourtiaceae, are effective in treating some skin diseases and leprosy (1). Most noteworthy historically are the effects of these oils or their acyl moieties on Mycobacteria (2). Recently, a more searching and systematic interest in the chemistry and pharmacologic effects of not only the natural oils, but of some of their derivatives, has developed (3).

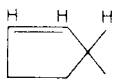
The seeds of *Hydnocarpus wightiana* contain 40% or more of triacylglycerols having ca. 85% content of therapeutically effective acyl moieties (4). The structure of the 3 most important fatty acids are:

Hydnocarpic acid



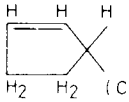
H_2 H_2 $(CH_2)_{10}COOH$ (16:1cy)

Chaulmoogric acid



H_2 H_2 $(CH_2)_{12}COOH$ (18:1cy)

Gorlic acid



H_2 H_2 $(CH_2)_6CH=CH(CH_2)_4COOH$ (18:2cy)

SCHEME I

It should be mentioned, however, that the gorlic acid of *H. wightiana* seed oil was found to be a mixture having 85.5% of fatty acid with a double bond in the $\Delta 6$ position and 14.5% with a double bond in the $\Delta 9$ position (4).

This report on some aspects of the autoxidation of esters of these fatty acids appears warranted for several reasons. First, because they are applied topically in the treatment of some skin diseases and leprosy, it is conceivable that their autoxidation, or resulting primary or secondary products, may play some important role, as yet unexplored, in their therapeutic effects.

Second, there appears to be some possibility that the esters of cyclopentenyl fatty acids on autoxidation form some endoperoxides, or other oxygenated 5-carbon carbocyclic derivatives that resemble some of the highly biologically active endoperoxides, prostaglandins and thromboxanes, for example, which are biosynthesized oxygenated 5-carbon carbocyclic derivatives of arachidonic and γ-dihomolinolenic acids. The possibility that biological activities exist in autoxidized esters of cyclopentenyl fatty acids therefore appears to merit further investigation.

Additionally, there is the somewhat more abstract scientific merit in learning how the autoxidation of these compounds, hitherto unexplored, resembles and differs from that of other natural and synthetic carbocyclic compounds, cyclic and noncyclic ethers and other presumably comparable organic compounds.

MATERIALS

In all critical stages of the preparation and

use of materials, conventional procedures to prevent harmful interference by oxygen were employed.

Methyl Esters of Cyclopentenyl Fatty Acids

Ground seeds of *H. wightiana* were extracted twice in a Soxhlet apparatus with purified hexane. The oil (I.V. 97.3 to 99.2) (5) was saponified by heating for ca. 1 hr with 10% KOH in aqueous methanol. The resulting mixture was extracted several times with diethyl ether; evaporation of the combined extracts yielded a residue of 0.19% nonsaponifiable material.

The soap-containing mixture was acidified with H_2SO_4, and the free fatty acids were then obtained in 3 extractions with petroleum ether-diethyl ether. The combined extracts were washed with water until neutral, dried over anhydrous Na_2SO_4 and evaporated under nitrogen.

The acids were converted to their methyl esters with an excess of methanol-H_2SO_4 (6). The reaction mixture was diluted with water and extracted several times with petroleum ether-diethyl ether. The extract was washed with water until neutral and dried over anhydrous Na_2SO_4, then evaporated under nitrogen.

The composition of the purified mixed methyl esters as determined by the gas liquid chromatography (GLC) methods described later, and expressed in % wt, was hydnocarpate 49.2, chaulmoograte 30.7, gorlate 10.4, palmitate 3.8, palmitoleate 0.9, stearate 0.8, oleate 3.2 and others 1.0.

After adding 20% by wt of methyl behenate to act as "pusher," the methyl esters were fractionally distilled under vacuum at a pressure of ca. 1 mm Hg. During the complete distillation, the pot temperature rose from 240 to 255 C, and the reflux temperature from 128 to ca. 177 C. During several such distillations, 20-25 fractions were collected. GLC analysis revealed that the combined C_{16} ester fractions consisted mainly of hydnocarpate together with small amounts of palmitate and palmitoleate. The combined C_{18} ester fractions consisted mainly of chaulmoograte and gorlate plus quite small amounts or traces of stearate, oleate, linoleate, linolenate and other esters.

The C_{16} and C_{18} ester fractions were converted to fatty acids as before and subjected to crystallization and several recrystallizations of 2-10% solutions in acetone at -5 to -60 C. The 3 important cyclopentenyl fatty acids were then converted individually to their methyl esters as before.

The identities and purities of the 3 principal final methyl esters were assessed by 3 methods. First, they were analyzed by adsorption-thin layer chromatography (TLC) and argentation-TLC (7).

Second, the esters were analyzed by gas-chromatography (8) using a Perkin-Elmer F22 gas chromatograph instrument equipped with a hydrogen flame ionization detector. Glass columns (2 m x 0.5 cm id) were packed with 10% GPC Apiezon L on Anakrom ABS (90-100 mesh) or with 10% Silar 5CP on Gas-chrom Q (80-100 mesh). The temperatures used were 230 and 220 C for the Apiezon and Silar columns, respectively. The carrier gas was pure nitrogen at a flow rate of 40 ml/min. The temperatures of the injection port and detector were 250 C.

Third, a new method developed in these laboratories was used to identify the methyl esters of cyclic fatty acids unambiguously which involves a combination of GLC with mass spectrometry (MS) of their pyrrolidides (9).

Using the methods described, the purities of the methyl hydnocarpate, chaulmoograte and gorlate used in the autoxidation studies were found to be > 99.3, > 99.6, and $> 99.2\%$, respectively.

Methyl Oleate and Linoleate

Methyl oleate and linoleate were purchased from Nu-Chek-Prep (Elysian, MN) and reportedly were $> 99\%$ pure. These levels of purity were verified by GLC.

Miscellaneous Materials and Analytical Reagents

All solvents and other chemicals used were of analytical grade.

EXPERIMENTAL PROCEDURES

Two main series of experiments were conducted. The principal differences were: (a) in the first series, the autoxidations were carried to ca. 30 meq per kg of the hydnocarpate and chaulmoograte at 3 temperatures, 40, 60 and 80 C and of gorlate at 80 C only, and provided information about the kinetics and products through the very earliest stages to a level of oxidation of ca. 0.4% of the sample where there were virtually no complications from secondary products formed from peroxide decomposition or by oxidation of peroxide decomposition products, and (b) in the second series, the autoxidations were carried to levels of ca. 300 or slightly more meq of peroxide per kg of each of the 3 esters, and methyl oleate and linoleate for comparative purposes, to provide more information about kinetics and more product for subsequent structure exami-

nations, using only one temperature of autoxidation, 60 C.

Oxygen Absorption

A standard form of Warburg equipment with 14 double manometers (Foto-Warburg) model VL 85, was employed (10) using a glycerol bath whose temperatures were controlled at 40, 60 and 80 C (± 0.5). Samples were shaken through an amplitude of 2.5 cm at ca. 120 times per min. The manometers contained Kreb's manometer fluid having a specific gravity of 1.03 g/ml at 20 C. The flasks and attached manometric systems had volumes of ca. 15 ml. Sample sizes usually were ca. 0.05 or 1 g; air volumes in flasks were corrected accordingly, using reliable sample specific gravity data available in the literature for the temperatures used (11).

In experiments where oxidation was carried to maximum levels of ca. 5%, the flasks were flushed with fresh air very briefly during manometer readings and adjustments to prevent appreciable diminution of partial oxygen pressure.

Initially, when the rate of oxygen absorption was so low it could be ignored, the samples were equilibrated with air by shaking for ca. 20 min before the systems (manometer and flask stopcocks) were closed.

Peroxide Values

Peroxides values were determined by the iodometric method described by Privett et al. (12) with minor modifications. Samples of 0.1 to 0.5 g were analyzed using solvents and reagents including 0.002 to 0.01 N sodium thiosulfate, all free of dissolved oxygen.

UV Absorption Measurements

UV absorption measurements of the initial and autoxidized samples dissolved in ethanol (95%, "Uvasol" for spectrophotometry, E. Merck AG) were made in the range from 220 to 310 nm. The dissolved samples were examined in a quartz cuvette having a path of 1 cm, using a PMQ II spectrophotometer (Carl Zeiss).

UV measurements of the KI-reduced autoxidation products extracted after peroxide value determinations were also made on all samples, including oleate and linoleate, autoxidized at 60 C only.

IR Absorption Measurements

The IR spectral absorption of the methyl esters of cyclopentenyl fatty acids and their autoxidation products obtained at 40, 60 and 80 C were measured in the range from 200 to 4000 cm^{-1}. In the second set of experiments, similar types of data were obtained for all samples including oleate and linoleate, at levels of autoxidation approximating 300 meq of peroxide obtained at 60 C only. In this case, UV measurements were also made on the KI-reduced product.

The samples were dissolved in a spectrophotometric grade of CCl_4 (E. Merck AG) to give a 1% solution (w/v) and, using a path of 1 mm, were examined with a Perkin-Elmer IR spectrophotometer Model 397.

Thin Layer Chromatography (TLC)

The autoxidation products of the 5 esters employed in this study, as well as their KI-reduction products, were examined by TLC on Silica Gel H (E. Merck), at a thickness of ca. 0.3 mm on 10 x 20 and 20 x 20 cm plates, using hexane-diethyl ether (80:25, v/v) as the solvent system. Visualization of the separated components was accomplished by spraying with aqueous KI and starch solutions, or by spraying with 50% H_2SO_4 and charring at 200 C for 15 min.

Identification of the Autoxidation Products

The KI-reduction products have been obtained in concentrated and purified form by repeatedly extracting the residues of the peroxide determinations with diethyl ether-hexane (1:1, v/v), followed by washing the combined extracts repeatedly with water, and then dehydrating with anhydrous Na_2SO_4 and evacuating the solvents.

RESULTS AND DISCUSSION

Much of the information was obtained in the set of experiments at 60 C in which the methyl esters of the 3 cyclopentenyl fatty acids, methyl oleate and linoleate, were autoxidized to peroxide levels from 300 to almost 400 meq per kg.

Kinetics

The first series of experiments, as anticipated, showed that the rate of oxygen absorption at low levels increased with increasing temperature by roughly 2 to 4 times for each 20 C increase in temperature. The rates also increased with time, indicating the autocatalytic character of the reactions. Figure 1 shows the rate of O_2 absorption by methyl hydnocarpate at 40, 60 and 80 C in these early stages. Data for methyl esters of the other 2 cyclopentenyl fatty acids are not given, because they were very much like those shown for methyl hydnocarpate.

In a preliminary experiment, the rate of O_2 absorption of methyl hydnocarpate in pure hexadecane suggested (inconclusively) that the rate of autoxidation was proportional to the sample concentration. Other aspects of the data indicate the rates of autoxidation increased in an essentially proportional manner with the partial pressure of O_2 to ca. 150 mm Hg, which is consistent with observations by Bateman et al. (13).

Interesting, but unexplainable on the basis of our data, was that the rate of O_2 uptake to the level of 30 meq of O_2 per kg was higher for chaulmoograte than hydnocarpate at 40 C, about the same at 60 C and lower at 80 C. The rates for methyl gorlate autoxidation at very low levels of autoxidation were ambiguous; the comparative rates of autoxidation among the methyl esters of the 3 cyclopentenyl fatty acids, in comparison to methyl oleate and linoleate, are more accurately reflected in Figure 2.

This figure shows clearly that at low levels of autoxidation, the rates of O_2 uptake of methyl esters of the 3 cyclopentenyl fatty acids lie between those of methyl linoleate and oleate at 60 C. The relatively high rate for methyl linoleate is readily explained by the well known high reactivity of a hydrogen atom in a methylene group between 2 double bonds. In the esters of cyclopentenyl fatty acids where 1 double bond is located in a carbocyclic structure, 1 of the hydrogens appears to be more readily detached than a hydrogen in the α-position relative to a double bond in a straight chain carbon structure. Because esters of the 3 cyclopentenyl fatty acids involved in this study have a double bond in the ring which is adjacent to the carbon atom that is involved in linking the straight chain to the ring structure, and because it is well known that hydrogen atoms attached to such tertiary carbon atoms are quite reactive, it is probable that the esters of these cyclic fatty acids should be more readily autoxidizable than methyl oleate. However, it is apparent that methyl gorlate, which has a second double bond in the 6,7-position, is more readily autoxidized than methyl hydnocarpate and may equal linoleate in its rate of autoxidation in later stages.

Initial Products of Autoxidation

Because it is apparent that the primary initial products of autoxidation are peroxides, the meq of O_2 absorption per kg of *autoxidized product* (rather than per kg of original esters) are plotted against the measured peroxide values for methyl hydnocarpate during autoxidation in the first set of experiments at 40, 60

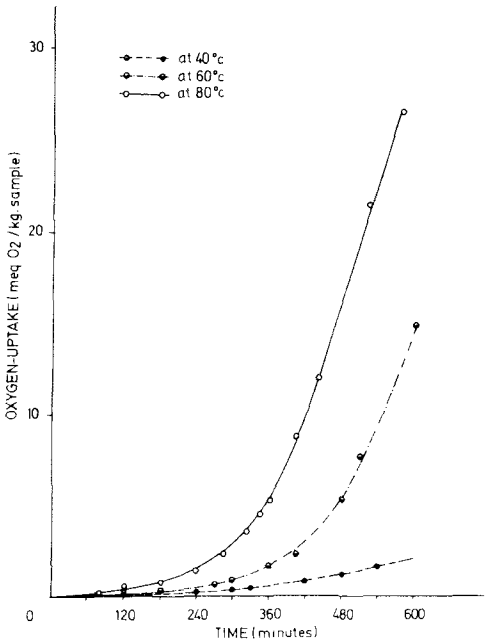


FIG. 1. Autoxidation of methyl hydnocarpate at 40, 60 and 80 C.

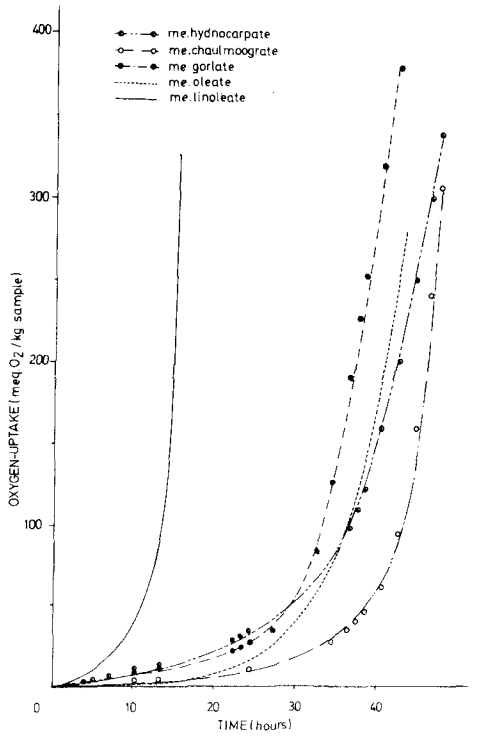


FIG. 2. Autoxidation of methyl esters of cyclopentenyl fatty acids, methyl oleate and linoleate at 60 C.

and 80 C in Figure 3. Because of the data for methyl chaulmoograte and gorlate follow the same pattern, qualitatively and essentially quantitatively, no figures are provided for the other 2 esters.

Clearly, the primary autoxidation products of the methyl esters of cyclopentenyl fatty acids are hydroperoxides, but from the beginning, secondary products are formed at rates that bear a constant ratio to the rates of peroxide formation. These products therefore are not formed by peroxide decomposition, because if they were so produced, their rate of formation would show an increase relative to the rates of peroxide formation as the autoxidation progressed to higher levels. This is consistent with similar findings by Lundberg and Chipault (14) in the autoxidation of methyl linoleate at various temperatures, and is completely verified by the results of our studies on the autoxidation of oleate and linoleate at 60 C. Also, our findings of increasing amounts of these secondary products at increasing temperatures are virtually the same as their findings in the case of methyl linoleate autoxidized at various temperatures.

On the basis of our observations on the kinetics and the initial autoxidation products of methyl esters of the 3 cyclopentenyl fatty acids, we make the following conclusions, which will be further substantiated in the section that follows: (a) the autoxidation of the methyl esters of cyclopentenyl fatty acids proceeds via a free radical chain mechanism that yields peroxides as the principal products; (b) secondary oxidation products not resulting from peroxide decomposition are formed in small but increasing amounts with increasing temperatures of autoxidation. Because they are formed in direct proportion to the peroxides, it is probable that oxygenated free radicals are involved in their formation; (c) the kinetic data, and data that will follow, show there are some kinetic, mechanistic and structural differences in the products as compared to those obtained in the autoxidation of methyl oleate and linoleate.

It should be mentioned that, in the experiments with methyl esters of the 3 cyclic fatty acids, organoleptic developments ("rancidity") appeared at various peroxide levels between 30 and 300 meq/kg. These must have stemmed from minute amounts of secondary oxidation products formed by peroxide decomposition and by further oxidation of the product, because most of the early products, even at peroxide levels of 300 meq/kg, were apparently high molecular weight nonvolatile secondary products. The development of very small amounts of organoleptically offensive compounds at comparably low levels of autoxidation of unsaturated triacylglycerols in food fats is well known (15).

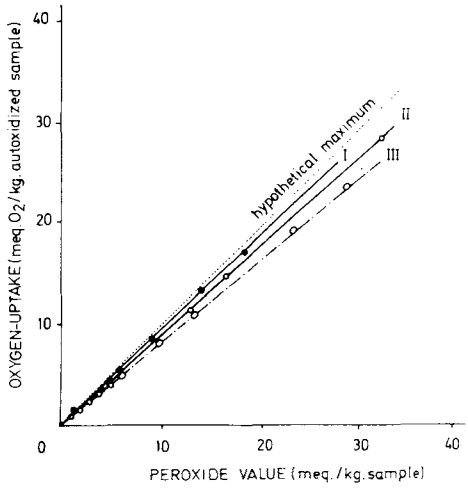


FIG. 3. Peroxide values in relation to oxygen uptake for methyl hydnocarpate at 40 C (I), 60 C (II) and 80 C (III).

UV Absorption

Measurements of the UV absorption of the methyl esters of cyclopentenyl fatty acids autoxidized to levels of 300 or more meq of peroxide per kg at 60 C yielded some additional information. Methyl hydnocarpate and chaulmoograte showed only small changes in absorption in the region between 210 and 310 nm, but methyl gorlate, autoxidized to a peroxide value of 380 meq/kg, showed a remarkable increase in absorption at 232 nm (Figure 4) comparable to that found for methyl linoleate oxidized to the same level by Lundberg and Chipault (14), and by us in the second set of experiments.

This strongly suggests methyl gorlate is converted primarily to a conjugated diene hydroperoxide (or some peroxide with similar UV absorption characteristics).

Figure 4 also reveals the development of some absorption at a point slightly above 250 nm, which is attributed to secondary products of unknown identity, possibly enolized unsaturated ketones. We have also found a slight increase in absorption in this region in autoxidized methyl oleate, which our IR data suggest may result from an enolized ketone. Others have suggested that increased UV absorption in the conjugated triene region (270-275 nm) in the case of autoxidized methyl linoleate is caused by enolized conjugated diene ketone

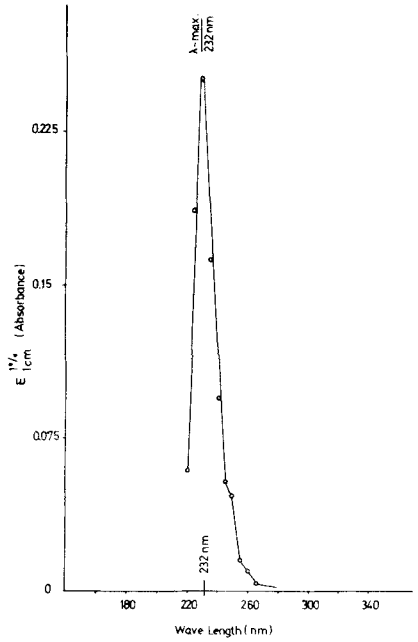
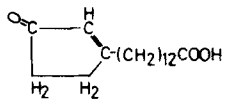


FIG. 4. UV absorption of methyl gorlate to a peroxide value of 380 meq/kg autoxidized at 60 C.

formed, but not by peroxide decomposition, at a position relative to the double bonds (15). Sapucainha oil has been reported to contain ketochaulmoogric acid (16), and it appears



SCHEME II

highly probable that this could be formed by oxidative attack at the number 1 carbon of the cyclopentene ring to form a free radical, which may then resonate to shift the double bond and yield a free radical at the number 3 carbon; this radical could then react with oxygen and yield some ketone as one of the products.

IR Absorption

The IR spectra of methyl hydnocarpate and chaulmoograte are essentially the same, and undergo virtually no change after autoxidation to ca. 30 meq of peroxide per kg employed in the first set of experiments involving temperatures of 40, 60 and 80 C. Important differences developed, however, in experiments with gorlate.

In all 3 esters, vibrational absorption of the tertiary CH linkage at position 1 of the cyclopentene ring, which is very weak at 2890 cm^{-1} (3.46 μ), is only slightly affected by autoxidation.

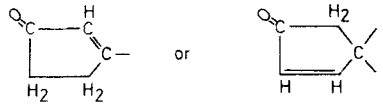
The *cis* double bond in the cyclopentene ring shows some absorption at 700 cm^{-1} (14.3 μ) and the intensity of this peak decreases markedly in all 3 esters at peroxide levels of 30 meq per kg, and almost disappears at peroxide values of 300-400 attained at 60 C.

The methyl esters of the cyclic fatty acids also show a peak double bond absorption at ca. 1650 cm^{-1} (6.06 μ) and no appreciable changes were found after autoxidation. However, the double bonds in the ring of all 3 of the cyclic methyl esters showed another significant absorption at 3050 cm^{-1} (3.28 μ), which disappeared in all cases upon autoxidation to 300-400 meq of peroxide per kg at 60 C.

Methyl gorlate showed an additional double bond absorption at ca. 3000 cm^{-1} (33.3 μ) attributable to the double bond in the straight carbon chain position of the molecule, and this completely disappeared upon autoxidation to a peroxide level of 380 meq per kg.

In all 3 instances, after autoxidation to ca. 30 meq of peroxide per kg, at 40, 60 and 80 C, there was an increase in intensity of the triplet absorption bands between 3450 and 3600 cm^{-1} (2.9-2.78 μ), indicating development of OH groups. The absorption was not great and apparently indicated development of an intermolecular water-bridge.

Also, in all experiments, an absorption appeared at 1725 cm^{-1} (5.8 μ), which is a carbonyl stretching band indicating the formation of aldehyde and/or ketone, which were independant of the carbonyl in the carboxylate group at 1740 cm^{-1} (5.75 μ). Conjugation of the noncarboxylate carbonyl with an ethylene group decreases the vibrational frequency below that of a ketone (1740-1750 cm^{-1}, or 5.71-5.75 μ). On the other hand, compounds with a keto group adjacent to the double bond, such as Scheme III, have been found to have



SCHEME III

vibrational frequencies at 1725 cm^{-1}, (5.8 μ) as we found in the autoxidized methyl esters of cyclopentenyl fatty acids. The first of the foregoing structures is like that reported by Paget in Sapucainha oil (16). This strongly suggests that a principal point of oxidative attack is at the tertiary carbon in the cyclo-

pentene ring and, that the nonperoxidic secondary oxidation products (not formed by peroxide decomposition) are ketones like the first of the 2 structures in Scheme III, formed after resonance of the initial free radical formed at the tertiary carbon involving a double bond shift and subsequent formation by oxidation at the number 3 carbon of the cyclopentene ring.

Autoxidized methyl gorlate shows a band at ca. 1610 cm^{-1} (6.21 μ) indicating the presence of additional conjugated double bonds. It is well known that conjugated dienes without a center of symmetry show 2 absorption bands, one near 1600 cm^{-1} (6.25 μ) and the other near 1650 cm^{-1} (6.06 μ). The first of these bands is most useful in determining conjugation since the second overlaps absorption by single double bonds in the 1660-1640 cm^{-1} region (6.0-6.1 μ).

The IR absorption of autoxidized methyl gorlate supports our findings of strong UV absorption in the 232 nm region resulting from conjugated diene.

The autoxidized methyl esters of the straight-chain fatty acids, oleic and linolenic acids, showed more or less a similar picture as with the methyl esters of cyclic fatty acids. Conjugated diene was more detectable in the methyl linoleate than in methyl oleate.

The IR spectra of all the reduced peroxides showed increased OH absorption and loss of peroxide absorption for the methyl esters of all 3 cyclopentenyl fatty acids.

Also, increased IR absorption was found as a low frequency weak band at 960 cm^{-1} (10.42 μ), indicating some *trans* double bonds were formed as anticipated with the shifting double bonds during autoxidation of the esters.

Thin Layer Chromatography

A thin layer chromatogram of the methyl esters of cyclic and straight-chain fatty acids autoxidized to peroxide values of 300-400 meq per kg at 60 C, together with appropriate unoxidized standards, is shown in Figure 5. The chromatogram was sprayed with aqueous KI and starch solutions or with 50% H_2SO_4 followed by charring.

All the unoxidized materials have the highest and essentially the same R_f values. The next lower clearly distinguishable spots in I, II, III, V and VI of the chromatogram represent the peroxides; in chromatograms of the reduced products, these spots are no longer present. Clearly, the R_f values for the peroxides of methyl oleate and linoleate are lower than those for the peroxides of methyl esters of cyclic acids.

Although not evident in Figure 5, but easily discerned in other chromatograms, 2 clearly differentiated peroxide spots appear in the case of methyl gorlate (III), representing 2 kinds of peroxides as might be anticipated from the presence of 2 double bonds, one in the cyclopentene ring and the other in the straight chain portion of the molecule.

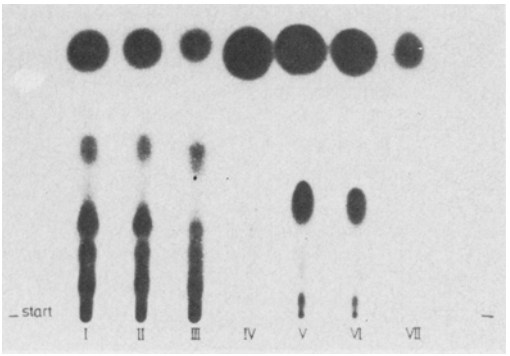


FIG. 5. Thin layer chromatogram of the autoxidized methyl esters of cyclopentenyl fatty acids, methyl oleate and linoleate at 60 C. I: autoxidized methyl hydnocarpate. II: autoxidized methyl chaulmoograte. III: autoxidized methyl gorlate. IV: methyl chaulmoograte, V: autoxidized methyl oleate. VI: autoxidized methyl linoleate. VII: methyl stearate (Silica Gel H; 0.3 mm; hexane:diethyl ether = 80:25, v/v).

Although the intensity of the spots in Figure 5 is of no significance quantitatively, it appears from this figure that a greater variety of secondary autoxidation products with relatively low R_f values are formed in the autoxidation of methyl esters of cyclopentenyl fatty acids, such as ketones and other side products, but this is not apparent in the chromatograms of the reduced autoxidation products. Thus, again the differences observed in the spots for autoxidation products with low R_f values in Figure 5 may simply reflect an influence of the carbocyclic ring.

Structure of the Initial Autoxidation Products

Any extensive discussion about structures of the initial products of the autoxidation of methyl esters of cyclopentenyl fatty acids is unwarranted until GLC-mass spectral data are available. It may be said based on the preceding results that the principal products are peroxides, probably hydroperoxides, formed by mechanisms closely related to those involved in the autoxidation of straight-chain unsaturated lipids. Small amounts of secondary oxidation products, which appear to be ketones, are formed, not by peroxide decomposition, but probably via oxygenated free radicals of the cyclic esters.

Of particular interest is the formation of a high proportion of conjugated diene during the autoxidation of methyl gorlate, but because several speculative mechanisms could be proposed, it is advisable to defer further consideration until mass spectral data become available.

The formation of endoperoxides has not been ruled out by any of the foregoing evidence, and thus has potential from the standpoints of several chemical and biological considerations. The cyclopentene structure and the autoxidation products may closely resemble those of other highly biologically active compounds that develop in mammals in vivo from arachidonic and γ-dihomolinolenic acid (17).

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Desaturation of Positional and Geometric Isomers of Monoenoic Fatty Acids by Microsomal Preparations from Rat Liver

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ABSTRACT

A range of *cis*- and *trans*-monoenoic fatty acids was tested as substrates for desaturation in microsomal preparations from rat liver. *Trans*-monoenoic acids were generally desaturated in the Δ9 position to the same extent as stearic acid. Acids with Δ7-*trans*- and Δ11-*trans*-olefinic unsaturation produced Δ7-*trans*,9-*cis*- and Δ9-*cis*,11-*trans*-conjugated dienoic acids, respectively, but the Δ8-*trans*- and Δ10-*trans*-monoenoic acids did not give Δ8,9- or Δ9,10-allenes. Of the *cis*-monoenoic acids examined, only those with double bonds at or beyond the Δ14 position gave any measurable Δ9 desaturation. When Δ9 desaturation of long chain saturated acids was inhibited by adding sterculic acid, these saturated acids were desaturated at the Δ5 and Δ6 positions. Many of the monoenoic acids tested were also desaturated at the Δ5 and/or Δ6 positions, although the percentage conversions were always low. Δ9-*cis*,11-*trans*-, Δ9-*cis*,12-*trans*- and Δ9-*cis*,13-*trans*-dienoic acids, produced in situ by Δ9 desaturation of the corresponding monoenoic acids, were extensively desaturated in the Δ6 position. These results are discussed in terms of: (a) the various models proposed to explain the substrate specificities of the desaturases, and (b) the metabolism of unnatural fatty acids ingested from dietary sources.

INTRODUCTION

The desaturases that introduce Δ9-*cis*, Δ6-*cis* or Δ5-*cis* double bonds into fatty acyl chains have been well characterized in animal microsomal preparations. Of these, the Δ9 desaturase has been the most extensively studied, and its recent purification from rat liver by Strittmatter et al. (1) has enabled his group to reconstitute an active, purified Δ9 desaturase system for further study (2) from the desaturase protein, cytochrome b_5 reductase, cytochrome b_5 and phospholipid vesicles. Enzyme activity requires NADH and molecular oxygen; acyl-CoA thioesters are the substrates. A fairly precise model for the enzyme-substrate binding site has been proposed for the Δ9 desaturase (3,4), based on the absolute stereochemistry of hydrogen abstraction (5,6), on the exact positional specificity of desaturation and on substrate specificity studies (3,7). The position of desaturation is believed to be under carboxyl-end control. The hydrocarbon chain is accommodated in a deep enzyme "cleft" of hydrophobic nature, with a very tight spatial constraint between C-5 and C-15 bringing the 9*D* and 10*D* hydrogen atoms to the active center. The mechanism for hydrogen abstraction still is unknown. This model implies that the acyl chain is constrained to a predominantly extended conformation, i.e., carbon-carbon bonds in an antiperiplanar (*trans*) conformation. To our knowledge, no *trans* and only a few *cis* long chain monoenoic acids have been tested as substrates for Δ9 desaturation (3,8,9). With the exception of 18:1 (12c) in microsomal preparations from hen liver and goat mammary gland (but not rat liver), these *cis*-monoenoic acids were not desaturated.

This work tested an extensive range of positional isomers of *cis*- and *trans*-monoenoic acids in microsomal preparations from rat liver in order to assess the proposal that only *trans*-unsaturated acids would undergo Δ9 desaturation according to the model advanced by Brett et al. (3). We tested a large number of novel substrates and emphasized product identification. In many instances, Δ5 and/or Δ6 desaturation of the substrates and of the dienoic acids produced in situ by Δ9 desaturation were observed. These unexpected Δ5 and Δ6 desaturations are discussed in terms of the approaches they suggest for further studies on the substrate specificity of these two desaturases. Present models explaining the substrate specificity of Δ5 desaturation (4) and Δ6 desaturation (4,10) are poorly defined when compared to the model for Δ9 desaturation (3,4). Finally, this work has relevance to studies on the metabolism of fatty acids with *trans*-unsaturation, a subject recently reviewed by Houtsmuller (11).

MATERIALS AND METHODS

Fatty Acids

[1-^{14}C]Palmitic, stearic, oleic, linoleic and α-linolenic acids (sp act 50 to 60 Ci/mol) were

Fatty acid nomenclature follows these examples: palmitic acid, 16:0; elaidic acid, 18:1 (9*t*); and linoleic acid, 18:2 (9*c*,12*c*).

purchased from the Radio-chemical Centre, Amersham, England. The monoenoic [1-^{14}C] acids were prepared by the chain extension of alkenyl mesylates or chlorides with potassium [^{14}C] cyanide (sp act 60 Ci/mol) according to the scheme outlined in Figure 1.

Isomeric heptadecen-1-ols (*cis/trans* mixtures), **II**, prepared by the anodic decarboxylation of *cis/trans* mixtures of octadecenoic acids, **I**, according to the method of Gunstone et al. (12), were converted to the mesylates, **III**, by the method of Crossland and Servis (13). 1-Chloro-alkynes, **IV**, were synthesized by the coupling of alkyl bromide to ω-chloroalk-1-ynes in the presence of sodamide in liquid ammonia (14), and were reduced to *cis*-1-chloroalkenes, **V**, by hydrogenation in the presence of Lindlar catalyst partially poisoned with quinoline (15). Stereomutation of small portions of the 1-chloroalkenes was accomplished using oxides of nitrogen (16), with polar by-products being removed by thin layer chromatography (TLC). Alkenyl [^{14}C] nitriles, **VI**, were obtained by heating excess alkenyl mesylate or chloride (15-20 μmole) with K^{14}CN (300 μCi, 5 μmole) in dry DMSO (0.3 ml) in a sealed tube for 16 hr at 85-90 C or 125-130 C, respectively. The nitriles were converted to their methyl esters, **VII**, by methanolysis (17). The methyl [1-^{14}C] alkenoates were separated into *cis*- and *trans*-geometric isomers by argentation thin layer chromatography (Ag^+ TLC), characterized and the esters saponified. Gas liquid chromatography (GLC) (mass and radiosiotope detection) of the esters indicated greater than 90% mass purity and greater than 95% radiochemical purity in all cases, while von Rudloff oxidative cleavage (18) showed no double bond migration (<1%) to have occurred.

The following [1-^{14}C] monoenoic acids were prepared for this study by (a) anodic decarboxylation: 18:1 (8*c*), (8*t*), (10*c*), (10*t*), (11*c*), (11*t*), (14*c*), (14*t*), (15*c*), (15*t*); and by (b) synthesis from acetylenic compounds: 16:1 (5*t*), (7*t*), (7*c*), (8*c*), (8*t*); 17:1 (5*t*), (12*t*), (12*c*); and 18:1 (5*t*), (5*c*), (13*t*), (13*c*). [1-^{14}C] Elaidic and linelaidic acids were prepared by stereomutation of methyl [1-^{14}C]-oleate and methyl [1-^{14}C] linoleate (16), and the products purified by Ag^+ TLC prior to saponification.

The urea adduct of methyl sterculate was donated by Dr. A.R. Johnson, C.S.I.R.O. Division of Food Preservation, Ryde, N.S.W., Australia. Sterculic acid was released from the adduct by the method of James et al. (19).

Enzyme Preparation

Rats of the Colworth-Wistar strain, fed for at least three months after weaning on a diet containing (by weight) 72% sucrose, 20% casein, 2% hardened coconut oil, and essential vitamins and salts, showed symptoms of essential fatty acid deficiency. The animals were killed by cervical fracture and their livers were excised, minced and rinsed with buffer solution. This and all subsequent manipulations were carried out at 5 C using a 0.3 M sucrose, 3 mM EDTA, 0.2 M sodium phosphate buffer solution at pH 7.3. The livers were homogenized in two volumes of buffer solution, and the resulting homogenate was centrifuged at 1,500 g for 15 min to remove cell debris and the fat pad. The postmitochondrial supernatant, obtained by spinning at 15,000 g for 30 min, was further centrifuged at 100,000 g for 1 hr. The 100,000 g pellet and some residual supernatant (ca. 1/10 of the total) were resuspended in buffer solution to give the unwashed

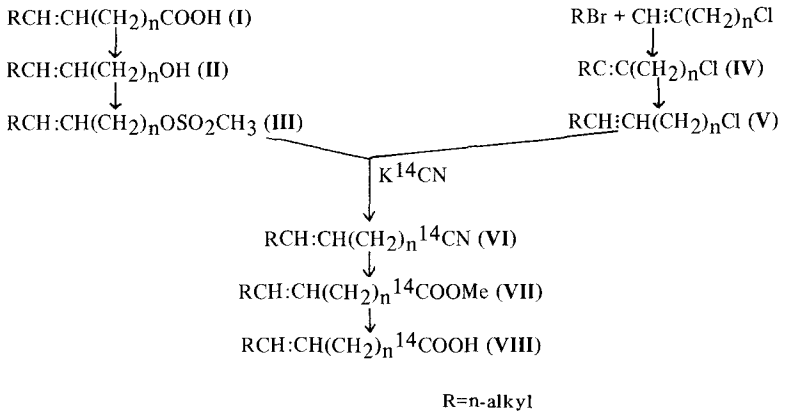

FIG. 1. Overall scheme for the synthesis of monoenoic [1-^{14}C] fatty acids (**VIII**).

microsomal fraction used in this study. Protein concentrations were estimated using the Biuret assay (20) with bovine serum albumin as a calibrant.

Incubations

Each incubation contained 1 ml of the enzyme preparation and 1 ml of the substrate solution. The latter was made by sonicating the [1-^{14}C] fatty acid (ca. 1.2 μCi) in 1 ml of distilled water plus two drops of a 10% (w/v) aqueous K_2CO_3 solution. The total incubation volume (2.5 ml) contained the following concentrations of substrate, protein and cofactors: [1-^{14}C] fatty acid, 8 μM; microsomal protein, ca. 1.6-2.5 mg ml^{-1}; 100,000 g supernatant protein, ca. 1.3-1.5 mg ml^{-1}; ATP, 4.3 mM; CoASH, 52 μM; NADPH, 140 μM; NADH, 340 μM; and $MgCl_2$, 4 mM. The reaction was started by the addition of the substrate solution to the enzyme preparation containing the necessary cofactors (at pH 7.3), and the incubation vial was gently agitated at 37 C in air. The reaction was terminated after 1 hr by the addition of 5% (w/v) methanolic KOH (10 ml), and the mixture was allowed to stand overnight at 45 C. The fatty acids were recovered from this saponification by acidification and extraction into petroleum ether, and they were converted to their methyl esters using diazomethane. For analysis of the labeled lipid classes, aliquots were removed from the incubation medium just prior to the termination of the reaction, and these were added to two volumes of methanol and rapidly frozen (-20 C).

Lipid Analysis

Neutral lipids were separated on Silica Gel TLC plates using a petroleum ether (bp 40-60 C)-diethyl ether-acetic acid, 80:20:1 (v/v/v) solvent mixture for elution. Polar lipids were separated on Silica Gel H impregnated with 2% (by wt) potassium oxalate with a chloroform-methanol-water, 45:35:10 (v/v/v) solvent system. Unsaturated methyl esters were examined by Ag^+ TLC (5% silver nitrate by weight, in Silica Gel G) with petroleum ether-diethyl ether mixtures. Radioactive bands were located by autoradiography, and the silica gel was scraped directly into a scintillation medium for counting the ^{14}C-activity.

For radioisotope detection by GLC (subsequently referred to as radio-GLC) an instrument similar to that described by James and Piper was used (21). Fatty methyl esters were analyzed on a 10% diethylene glycol succinate (DEGS), 9 ft packed column. Quantitation of the radioactivity trace was by triangulation.

The double bond position of [1-^{14}C] unsaturated acids was determined by a microscale modification of the von Rudloff permanganate-periodate oxidation (18). After esterification with diazomethane, the ^{14}C-labeled diester fragments were analyzed by radio-GLC, with co-injection of unlabeled methyl diesters of dicarboxylic acids as standards. For identification of ^{14}C-products where the new double bond was inserted between an existing double bond and the methyl end of the substrate, partial reduction of the diene with hydrazine (22) followed by Ag^+ TLC separation of the monoene fraction preceded von Rudloff oxidation.

The distribution of radioactivity between substrate and product acyl groups was measured by two methods. An aliquot of the ^{14}C methyl esters was analyzed by radio-GLC, whereas another aliquot was examined by Ag^+ TLC. Good agreement between the two methods was obtained for the percentage desaturation of the ^{14}C substrate, but at low percentage conversions ($\leqslant$ 5%) Ag^+ TLC was preferred for quantitation. For very low conversions (1-2%), the product had first to be isolated by preparative Ag^+ TLC before a distinct peak could be observed by radio-GLC. The detection limit for desaturation was ca. 1%. Every ^{14}C product was identified by three criteria: its equivalent chain length (ECL) value on a 10% DEGS packed column; its R_f value on Ag^+ TLC plate; and the position of the new double bond as located by von Rudloff oxidation.

RESULTS

For this study, free [1-^{14}C] fatty acids (8 μM) were incubated for a 1 hr period with moderate amounts of microsomal protein (2 mg ml^{-1}) in the presence of a large stoichiometric excess of cofactors for activation and desaturation. The percent conversions measured logically represent the end point of an incubation with nonsaturating amounts of substrate, and cannot be considered to represent maximum velocities. These conditions were used to facilitate high yields of products for identification. The microsomes were from rats fed for several months after weaning on a high sucrose diet containing only a small amount of saturated fat, since it is well-documented that essential fatty acid deficiency stimulates both Δ9 and Δ6 desaturations and that a high carbohydrate diet also stimulates Δ9 desaturation (23,24).

Table I shows the microsomal preparation contained highly active Δ9, Δ6 and Δ5 desatu-

TABLE I

Desaturation of the Natural Substrates in Microsomal Preparations from Rat Liver[a]

Conversion	Percent Conversion (this study)	nmole min^{-1} mg $protein^{-1}$
16:0→16:1(9*c*)	77	2.1
18:0→18:1(9*c*)	55.5	3.3
16:1(9*c*)→16:2(6*c*,9*c*)	10.5	0.15
18:1(9*c*)→18:2(6*c*,9*c*)	5	0.15
18:2(9*c*,12*c*)→18:3(6*c*,9*c*,12*c*)	13.5	1.0
18:3(9*c*,12*c*,15*c*)→18:4(6*c*,9*c*,12*c*,15*c*)	37.5	---
20:3(8*c*,11*c*,14*c*)→20:4(5*c*,8*c*,11*c*,14*c*)	65.5	0.8

[a]A comparison of the percent conversions measured in experiment A (see Results, third paragraph) and maximum velocity measurements made by Bernert and Sprecher (25), who incubated 150 nmoles of the free fatty acid with 5 mg of microsomal protein plus the necessary cofactors for 3 min.

rases. A comparison is made with the maximum velocity measurements obtained by Bernert and Sprecher using liver microsomes from rats that were deficient in essential fatty acids (25). The microsomal preparation gave a clean reaction. Recoveries of radioactivity after saponification and treatment with diazomethane were of the order of 70% or greater, whereas TLC showed this ^{14}C radioactivity was associated with the fatty methyl ester band (>90%) and not with oxygenated fatty esters or other metabolites. No chain elongation was observed.

The results are organized around the following scheme: first, two sets of incubations, A and B, are reported. B contains the most extensive range of novel substrates tested for any single microsomal preparation and is discussed at length. Several additional experiments were performed using only parts of the available range of substrates, but these are not reported since the levels of desaturation observed were similar to experiments A and B. Second, to obtain a complete picture of the incubation of an individual substrate, Tables II and III must be used together. For example, Table II shows that 18:1 (13*t*) was both Δ6 desaturated (1.5%) and Δ9 desaturated (51.5%). However, 27% of the 51.5% 18:2 (9*c*,13*t*) produced was subsequently desaturated at the Δ6 position (Table III). The actual product distribution at the end of the incubation was 18:1 (13*t*), 41%; 18:2 (6*c*,13*t*), 1.5%; 18:2 (9*c*,13*t*), 42%; and 18:3 (6*c*,9*c*,13*t*), 15.5%.

Δ9 Desaturation

Table II shows all the monoenoic [1-^{14}C] fatty acids that were incubated with rat liver microsomes. The following general groupings of monoenoic acids as substrates for Δ9 desaturation can be made: (a) no Δ9 desaturation of Δ8 and Δ10 monoenoic acids was detected; (b) all other positional isomers of the *trans*-monoenoic acids were extensively Δ9 desaturated; (c) the Δ9 desaturation of Δ13 to Δ15 *cis*-monoenoic acids occurred, but the conversions were very low, and (d) *cis*-monoenoic acids with the double bond before the Δ13 position were not substrates for the enzyme.

The chain length of the substrates was generally C_{18}, but some C_{16} and C_{17} monoenoic acids were also used. Δ9 Desaturation of the C_{16}, C_{17}, and C_{18} series of saturated and Δ5 *trans*-monoenoic acids indicated chain length effects over this range would not be too great and therefore should not affect the above (a)-(d) grouping.

In several cases, the substrates were also desaturated at the Δ5 and/or Δ6 positions. However, the removal of ^{14}C-substrate by these competing reactions was small (≤15%). Thus, corrections applied to the percentage Δ9 desaturation values given in Table II to allow for such competition are minor and do not affect the general conclusions drawn from the data.

The chromatographic properties of the *cis,trans*-conjugated dienoic acids produced were particularly distinctive. A comprehensive tabulation of ECL and Ag^+ TLC R_f data for the substrates and their desaturated products is given elsewhere (26). ECL values of 18.2 for 16:2 (7*t*,9*c*) and 20.35 for 18:2 (9*c*,11*t*) on a DEGS column were considerably greater than those for nonconjugated dienoates of the same chain length (27), whereas on Ag^+ TLC the conjugated dienoates eluted faster than oleate (28). The Δ9 desaturation of 18:1 (8*t*) and 18:1 (10*t*) would have given 8,9- and 9,10-allenes, respectively. These compounds have an ECL on a DEGS column similar to that of lino-

TABLE II

[1-^{14}C] Fatty Acids Tested As Substrates for Desaturation in Microsomal Preparations from Rat Liver[a]

Saturated or *trans*-monoenoic substrates	Percent desaturation			*Cis*-monoenoic substrates	Percent desaturation		
	Δ9	Δ6	Δ5		Δ9	Δ6	Δ5
16:0	77	1	0				
17:0	65.5	1	0				
18:0	55.5	0	0				
16:1 (5*t*)	69.5	0	0				
17:1 (5*t*)	73	0	0				
18:1 (5*t*)	74.5	0	0				
18:0	55	0	0				
18:1 (5*t*)	80	0	0	18:1 (5*c*)	0	0	0
16:1 (7*t*)	23	0	0	16:1 (7*c*)	0	0	0
18:1 (8*t*)	0	5.5	0	18:1 (8*c*)	0	0	7
16:1 (8*t*)	0	17	0	16:1 (8*c*)	0	0	1
18:1 (9*t*)	0	0	7.5	18:1 (9*c*)	0	4	0
18:1 (10*t*)	0	10	5.5	18:1 (10*c*)	0	0	0
18:1 (11*t*)	62[b]	5	0	18:1 (11*c*)	0	1	1
17:1 (12*t*)	14[b]	8	0	17:1 (12*c*)	0	5.5	0
18:1 (13*t*)	57.5[b]b	1.5	0	18:1 (13*c*)	1	1	0
18:1 (14*t*)	41.5[b]	0	10	18:1 (14*c*)	6	3.5	0
18:1 (15*t*)	48.5[b]	0	10	18:1 (15*c*)	4	4.5	0

[a]The upper portion of this table represents experiment A, the lower portion experiment B. Consult the third paragraph of Results for a full explanation.

[b]These products were subsequently Δ6 desaturated (Table III).

TABLE III

The Δ6 Desaturation of Some Dienoic Acids[a]

Substrate (added or generated in situ[b])	Percent Δ6 desaturation
18:2 (9*c*,11*t*) (in situ)	41
17:2 (9*c*,12*t*) (in situ)	80
18:2 (9*c*,12*t*) (added)	66
18:2 (9*c*,12*c*) (added)	16
18:2 (9*t*,12*t*) (added)	0
18:2 (9*c*,13*t*) (in situ)	27
18:2 (9*c*,14*t*) (in situ)	4
18:2 (9*c*,15*t*) (in situ)	4

[a]Results from experiment B. Consult the third paragraph in Results for a full explanation of this table.

[b]The Δ9-*cis*,11-15-*trans* isomers were produced during the incubation by Δ9 desaturation of the corresponding *trans*-isomers.

leate, and would have eluted with *trans*-monoenoates on Ag^+ TLC (28). No production of such allenic acids was detected.

Δ5 and Δ6 Desaturation

Many of the monoenoic acids tested were desaturated at the Δ5 and/or Δ6 positions (Table II). The percent conversions were low, but in several instances, competing Δ9 desaturation removed much of the substrate. The Δ6 desaturation of Δ8-*trans* isomers produced conjugated dienoates [16:2 (6*c*,8*t*) and 18:2 (6*c*,8*t*); ECL values of 17.9 and 20.25, respectively, on a DEGS column].

Several of the *cis,trans*-dienoic acids produced in situ by Δ9 desaturation were subse-

quently desaturated at the Δ6 position (Table III). The Δ9-*cis*, 11-*trans*-, Δ9-*cis*,12-*trans*- and Δ9-*cis*,13-*trans*-isomers were extensively desaturated when compared to linoleate, whereas the Δ9-*cis*,14-*trans*- and Δ9-*cis*,15-*trans*-isomers showed only marginal Δ6 desaturation. Linelaidic acid was not desaturated at the Δ6 position, but a small conversion (5%) to 18:3 (5*c*,9*t*,12*t*) was observed. Brenner has noted the absence of elaidic and linelaidic acid desaturation (10). However, this study shows small Δ5 desaturations of both acids are possible (Tables II and III). This agrees with the report of Lemarchal and Bornens on the Δ5 desaturation of elaidic acid (29). Radio-GLC detection of these Δ5 desaturations on a polar, packed column is not possible because of the low percent conversions and the unusually small differential in ECL value between substrate and product [18:1 (9*t*), ECL 18.6; 18:2 (5*c*,9*t*), ECL 19.0; and 18:2 (9*t*,12*t*), ECL 19.35; 18:3 (5*c*,9*t*,12*t*), ECL 19.75; compared with 18:1 (9*c*), ECL 18.6; 18:2 (6*c*,9*c*), ECL 19.2].

Lipid Incorporation

After a 1 hr incubation, the saturated and monoenoic acid substrates were all extensively esterified into lipids. The distribution of label into lipid classes was as follows: neutral lipids (mainly triacylglycerols, with smaller amounts of diacylglycerols), 20-50%; free fatty acids, 1-10%; polar lipids (principally phosphatidylcholine and phosphatidylethanolamine), 45-75%; and acyl-CoA, 2-5%. The distribution of substrate and product between the lipid classes was not measured.

Sterculic Acid Inhibition

The effect of sterculic acid inhibition on the isomer composition of the monoenoic acids produced from saturates was investigated. Coincubation of [1-^{14}C]heptadecanoic or stearic acid with an equimolar concentration of sterculic acid (8 μM) gave a marked inhibition of Δ9 desaturation, as expected (30). Heptadecanoic acid gave 19% 17:1 and stearic acid gave 10% 18:1, which compares with control levels of monoene formation in the absence of inhibitor of 80% and 55%, respectively. Von Rudloff oxidation of the monoene fraction recovered from Ag^+ TLC gave the following isomer distributions, expressed as a percentage of the total added ^{14}C-substate: 17:0 produced 79% 17:1 (9*c*) and 1% 17:1 (6*c*) in the absence of sterculate, and 7.5% 17:1 (9*c*), 8.5% 17:1 (6*c*) and 3% 17:1 (5*c*) when the inhibitor was added, whereas 18:0 produced 55% 18:1 (9*c*) in the absence of sterculate, and 1.5% 18:1 (9*c*), 1.5% 18:1 (6*c*) and 7% 18:1 (5*c*) with the inhibitor present.

DISCUSSION

Since the experiments were designed to facilitate product analysis, care must be taken in their interpretation. The percent desaturation will depend not only on the rate of desaturation, but also on the rate of activation of the free [1-^{14}C] fatty acid and the relative rates of removal of the acyl-CoA by the desaturases and acyl transferases. Thus, percent conversion is not synonymous with the rate of desaturation. This can be seen in Table I in a comparison of V_{max} vs. percent conversion data. However, we believe useful conclusions about substrate specificity can be made from judicious interpretation of the data. The results suggest strategies for further investigation of the substrate specificities of the desaturases. A full interpretation of the results awaits complete kinetic analysis.

The fatty acid-CoA ligase in washed microsomal preparations is reported to have a broad specificity with high maximal velocities (31, 32). All the ^{14}C acids in this study were extensively incorporated into lipids indicating activation. The use of low substrate concentrations (8 μM) compared to the endogenous levels of free fatty acids (ca. 90 μM; mainly 16:0, 18:0 and 18:1) in the enzyme preparation will probably reflect the in vivo competition between Δ9 desaturation and lipid incorporation more accurately when small quantities of such isomers are present in the diet than when using maximum velocity data from linear initial rate measurements at substrate saturating concentrations. It should be remembered that chain elongation will be another competitive reaction occurring at the endoplasmic reticulum in vivo.

The substrate specificity results for the Δ9 desaturase (Table II) fit the model advanced by Brett et al. (3), which is depicted in Figure 2a. In this static model, the acyl-CoA substrate is bound to the enzyme at the carboxyl end. The extended hydrocarbon chain of the substrate is held at the active center by a deep cleft of hydrophobic character with dimensions of 26 Å in length and 4 Å in width. A tight spatial constraint is imposed on the acyl chain between C-5 and C-15. The work of Enoch et al. (2) has pointed to the 9,10-*gauche* conformer of stearoyl-CoA as being the true substrate, and this is indicated in Figure 2a. The 9 *D* and 10 *D* hydrogen atoms will be in a position permitting their *cis*-elimination, producing oleoyl-CoA, which has a similar geometry to the bound substrate. Substituting a *trans*-double bond in place

of an antiperiplanar single bond in the extended hydrocarbon chain will cause minimum geometric perturbation of the alkyl chain (Figure 2b), so that the model predicts *trans*-monoenoic acids should be accommodated by the substrate binding site. However, a considerable perturbation results when a *cis*-double bond is introduced into the hydrocarbon chain. The substrate binding site model predicts it is unlikely for a *cis*-monoenoic acid to fit into the confined space of the cleft. This misfit can be viewed in either of two ways: the maintenance of the correct orientation of the methylene groups at the active center prevents the alkyl chain beyond the *cis*-double bond from fitting into the cleft (Figure 2c); or an acyl-chain (containing a *cis*-double bond) accommodated by the cleft requires unfavorable conformational changes in the remainder of the acyl-chain and incorrect alignment at the active center. Although this model is most readily envisaged in a static mode, a kinetic variation is also possible. As the CoA head-group of the substrate binds and the hydrocarbon chain "tumbles" into the hydrophobic binding site, each C-C bond is restrained to go through an antiperiplanar conformation before reaching the site. In such a dynamic model, however, a tight stearic control between C-1 and C-10 is still required to explain the absolute stereochemical and positional specificity of Δ9 desaturation. Brett et al. have already advanced the idea of a concerted conformational change in the protein structure which inserts or enfolds the hydrocarbon chain in a deep cleft (3).

The extensive Δ9 desaturation of the *trans*-monoenoic acids and the undetectable or minimal Δ9 desaturation of the corresponding *cis*-isomers argues for the validity of the above model. From their experiments with methyl-branched stearates, Brett et al. (3) proposed a region of only partial spatial restriction beyond C-15 as shown in Figure 2a. This may explain the appearance of low levels of Δ9 desaturation in the *cis*-monoenoates with their double bond beyond the Δ13 position. The range of positional isomers tested was incomplete. In particular, the Δ2, Δ3, Δ4 and Δ6 *cis*- and *trans*-positional isomers still require examination. The *trans*-isomers are expected to be good substrates for the Δ9 desaturase.

The lower levels of Δ9 desaturation of the Δ7 and Δ12 *trans*-monoenoic acids compared to the remaining *trans*-isomers tested (Table II) have yet to receive an adequate explanation. However, the Δ12 position does seem to be anomalous in its characteristics toward Δ9 desaturation, as in certain species (e.g., hen, goat) extensive Δ9 desaturation of the Δ12 *cis*-isomer can occur (3,8).

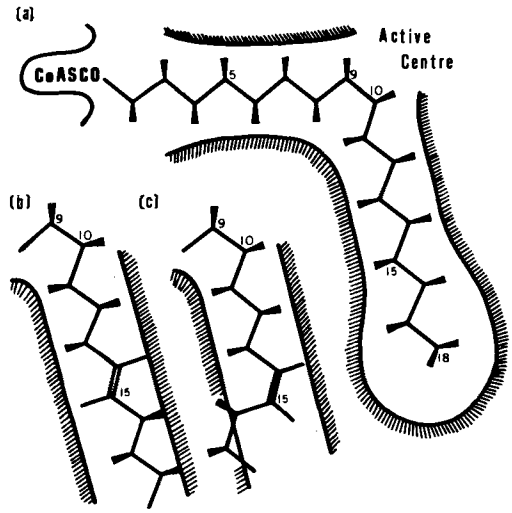

FIG. 2. (a) A schematic representation of the stearoyl-CoA: Δ9 desaturase enzyme binding site. (b) *trans*-double bond occupying the cleft. (c) *cis*-double bond occupying the cleft. The CoA binding site is shown by the single line, whereas the apolar surface of the enzyme, a deep groove or cleft constraining the methylene chain, is shown by the hatched line. Because of the two-dimensional representation, the C-9-C-10 bond appears as the synperiplanar conformer rather than the proposed *gauche* conformer. For further explanation, see Discussion.

An interesting feature was the production of conjugated dienoic acids. This is believed to be the first report of biosynthesis of conjugated polyenoic fatty acids in animals. In contrast, the Δ9 desaturation of Δ8-*trans* and Δ10-*trans* monoenoates to give allenes was not observed. Although these potential substrates can fit into the proposed enzyme binding site, hydrogen abstraction would involve breaking vinyl C-H bonds (D_{dissoc} = 452kJ mole^{-1}) in a different orientation to the more usual alkyl C-H bonds (D_{dissoc} = 423kJ mole^{-1}) and, moreover, the linear geometry of the allene formed would be incompatible with a required 9,10-*cis* geometry for the desaturated product. Therefore, the absence of allene formation was expected.

The inhibition of Δ9 desaturation by sterculic acid resulted in the unexpected Δ5 and Δ6 desaturation of saturated acids. The Δ6 desaturation of palmitate and stearate has been detected in immature rat brain before (33), but the Δ5 desaturation of saturated acids by animals is completely novel, although known in bacterial systems (34). This evidence, along with the Δ5 and Δ6 desaturations of novel monoenoic acid substrates (Table II) shows a Δ9 *cis*-double bond is not obligatory for Δ6

desaturation and Δ8 *cis*- or Δ11 *cis*-double bonds are not obligatory for Δ5 desaturation (4). However, it should be noted that the rates of desaturation of monoenoic and saturated substrates will probably be low in comparison to the rates of desaturation of the polyenoic substrates (Table I), but this needs to be checked experimentally for the novel substrates. A very interesting observation was the extensive Δ6 desaturation of dienoic acids with a Δ9 *cis*-double bond and a *trans*-double bond in the Δ11-Δ13 position (Table III). When the *trans*-double bond was removed to the Δ14 or Δ15 position, the level of Δ6 desaturation dropped dramatically. Brenner has already noted that there is a cooperative effect between adjacent double bonds which extensively enhances Δ6 desaturation, since ∝-linolenate is a better substrate than linoleate which is, in turn, a much better substrate than oleate (10). The preliminary results reported here confirm this hypothesis and suggest a Δ9-*cis*,-12-*cis*-pattern of unsaturation is not critical. For example, since Δ8-*trans*-monoenoic acids are desaturated, a dienoic acid such as 18:2 (8*t*,12*c*) might be a suitable substrate for Δ6 desaturation, since it is now known that the enzyme(s) can accommodate Δ8-*trans*- and Δ12-*cis*-oleifinic unsaturation and since these double bonds are adjacent. This isomer of linoleic acid is present in partially hydrogenated oils (11). Another line of investigation these results suggest is the mapping of acyl chain configurations which are acceptable to the Δ5 and Δ6 desaturases. For example, Δ8-*trans*-, Δ9-*cis*- and Δ10-*trans*-monoenoic acids were all Δ6 desaturated, whereas Δ8-*cis*-, Δ9-*trans*- and Δ10-*cis*-isomers were not. These data indicate that the acyl chain must lie with a configuration approximating to *trans, cis* and *trans* geometry in the Δ8, Δ9 and Δ10 positions, respectively. Thorough studies are required to substantiate and extend this preliminary evidence.

The natural unsaturated acids of vegetable and animal fats are almost exclusively of the *cis*-configuration. However, partial hydrogenation of unsaturated oils for cooking oils, shortenings and margarines can produce significant amounts of *trans*-unsaturated acids, with octadecenoates usually the major species. Double bond migration can also occur, such that a range of positional isomers of both *cis*- and *trans*-octadecenoates may be found (35-37). Biohydrogenation in the rumen also results in the formation of such unusual isomers, which can be ingested via the consumption of milk, beef and dairy products (38,39). *Trans*-unsaturated acids are deposited in the tissues, although differential absorption of isomers can occur (37). Lipid incorporation, catabolism and anabolism, absence of EFA activity and effect on the physical properties of lipid membranes of *trans*-unsaturated acids, have been recently reviewed by Houtsmuller (11). Only two substantiated instances of the desaturation of *trans*-unsaturated acids are known: the Δ5 desaturation of 18:1(9*t*) (29,40) and the Δ6 desaturation of 18:2 (9*c*,12*t*) (41-42). The novel demonstration of Δ9 desaturation of many isomeric *trans*-monoenoic acids is yet another example of how their biological behavior resembles that of saturated acids. The presence of *trans*-acids at the site of Δ9 desaturation (the endoplasmic reticulum) depends on diet, absorption, transport, lipid incorporation and oxidative catabolism relative to the more conventional fatty acids, but now their Δ9 desaturation, possibly followed by further desaturation and chain elongation, must be considered as one possible fate. Such subsequent metabolism usually has been ignored in feeding studies with unusual fatty acids.

ADDENDUM

While this paper was in press, our attention was drawn to similar work reported by M.M. Mahfouz, A.J. Valicenti and R.T. Holman (Lipids, in press, 1980). These workers have observed that for certain isomeric *trans*-monoenoic fatty acids, geometric isomerization of the original double bond can occur during or after desaturation. As we did not check the geometric configuration of the original double bond in the desaturated products, it is possible that geometric isomerization may have occurred. This must be borne in mind when examining our results and conclusions.

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Relative Susceptibility of Microsomes from Lung, Heart, Liver, Kidney, Brain and Testes to Lipid Peroxidation: Correlation with Vitamin E Content

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ABSTRACT

Rates of in vitro lipid peroxidation of microsomes and homogenates were found to vary widely among different tissues and species. In rats and rabbits, lung microsomes peroxidized at a 25- to 50-fold lower rate than liver, kidney, testes and brain microsomes. Heart microsomes peroxidized at a rate slightly greater than, but most similar to, lung microsomes. Comparison of tissue homogenates also revealed the unique resistance of lung and heart to lipid peroxidation. The ratio of vitamin E to peroxidizable polyunsaturated fatty acids in lung and heart microsomes was several-fold higher than in microsomes from the other tissues studied, which accounted for the relative resistance of lung and heart to lipid peroxidation. Liposomes of extracted rat lung microsomal lipid were also resistant to peroxidation and the amount of vitamin E contained in the lung lipid extract was sufficient to confer the same degree of resistance when incorporated into an equivalent amount of rat liver lipid. Higher rates of peroxidation in mouse lung microsomes relative to rabbit, rat and human lung microsomes were similarly correlated with a lower ratio of vitamin E to peroxidizable fatty acids in mouse lung microsomes. These data provide strong support for the role of vitamin E as the major cellular antioxidant, especially in the highly oxygenated tissues of heart and lung, and demonstrate the utility of the microsomal system in characterizing tissue differences in susceptibility to peroxidative membrane decomposition.

INTRODUCTION

Intracellular lipid peroxidation has been often hypothesized as a mechanism of action of toxic agents (1) and has been implicated as a degenerative mechanism underlying cellular aging (2) and certain disease states (3). Liver microsomes or reconstituted systems containing extracted liver lipid plus purified liver microsomal NADPH-cytochrome P-450 reductase have been most frequently employed in investigations of the mechanism of peroxidation of the fatty acyl moieties of membrane phospholipids (4,5). Little information is available, however, regarding the relative susceptibility of membranes from various tissues to undergo lipid peroxidation. The peroxidizability of lung is of particular interest since it is the point of entry for oxidant gases contaminating the atmosphere. Roback observed that over 50 times more thiobarbituric acid-reactive material was produced during incubation of liver homogenate than during incubation of lung or spleen homogenates (6). Willis and Recknagel recently reported lung microsomes were only 4% as active as liver microsomes in producing malondialdehyde, although the basis for the low activity of lung microsomes was not identified in that study (7).

The biological antioxidant, vitamin E, has been shown to afford protection against the injurious effects of nitrogen dioxide and ozone on experimental animals (8). In addition, numerous studies have indicated susceptibility to lipid peroxidation is greatly influenced by tissue levels of vitamin E (9). Bieri and Anderson (10) demonstrated that the ability of tissue homogenates to undergo lipid peroxidation in vitro was inversely related to the dietary vitamin E status of the animal. Similarly, other investigators have shown that liver microsomes (11-13) or mitochondria (14) isolated from vitamin E-deficient animals peroxidize at a faster rate in vitro than fractions from control animals. Peroxidation of liver microsomes was inhibited by dietary supplement of vitamin E (12,13) or when vitamin E was added directly to liver microsomal suspensions (11,15), which support the role of vitamin E as a membranous antioxidant.

Taylor et al. (16) have shown there is a high degree of variation in the content of vitamin E in subcellular fractions from different tissues, which indicates there may be considerable differences among tissues with respect to protection against peroxidative reactions. In these investigations, we have measured rates of in vitro lipid peroxidation in microsomes and homogenates from several different tissues and have compared the variation in these rates with the microsomal vitamin E content. Microsomes and homogenates from lung and heart showed a low rate of in vitro peroxidation compared to

the other tissues studied. This resistance of lung and heart to lipid peroxidation can be explained by relatively high levels of microsomal vitamin E in these tissues.

EXPERIMENTAL PROCEDURE

Materials

Distilled water was filtered and deionized in a system custom designed by W.E. Chaffee Co., Inc., an affiliate of Continental Water of Buffalo, NY. NADPH, ascorbic acid, $FeSO_4$, EDTA, cytochrome c and *D,L*-α-tocopherol were obtained from Sigma Chemical Company, St. Louis, MO. Thiobarbituric acid was a product of Eastman Chemicals, Rochester, NY. Fatty acid methyl ester standards were purchased from Nu-Chek-Prep, Elysian, MN. Rats were male Long Evans (200-300 g), mice were female BALB/c (20-30 g) and rabbits were male New Zealand albino (2-3 kg).

Methods

All animals received Agway laboratory chow (Rochester, NY) ad libitum until the time of sacrifice. Animals were sacrificed by intraperitoneal (ip) injection of sodium pentobarbital, the chest was opened and a cannula was inserted into the trachea. Heart, lungs and liver were perfused by injecting ice-cold 0.15 M NaCl into the right ventricle of the heart while simultaneously ventilating the lungs through the tracheal cannula. Lungs were excised and the parenchyma was separated from the visible bronchi and blood vessels and minced thoroughly with scissors. Hearts, livers, kidneys, brains and testes were removed and similarly minced. The minced tissues were washed several times in ice-cold buffered potassium chloride (0.15 M KCl, 5 mM Tris-maleate, pH 7.4) containing 1 mM EDTA and homogenized in the same, using a teflon-glass homogenizer. The homogenate was centrifuged successively at 300, 1600, 8000 and 30,000 x g for 10 min at each speed and the pellets discarded. The "cell-free homogenate" refers to the supernatant after centrifugation at 300 x g. Microsomes were obtained from the 30,000 x g supernatant by centrifugation at 100,000 x g for 1 hr. The microsomal pellet was washed by suspension in and resedimentation from buffered potassium chloride with no EDTA, resuspended in the same type of solution and stored in liquid nitrogen. Lipid peroxidation in these EDTA-treated microsomes has been shown to be absolutely dependent on the concentration of added free ferrous iron (17). Protein concentrations were determined by the Lowry method (18); NADPH-cytochrome c reductase was assayed spectrophotometrically, as described by Williams and Kamin (19) and the initial rates measured were linearly proportional to time of incubation and amount of protein added over the ranges used.

Lipid peroxidation was measured by quantitation of malondialdehyde formed during the incubations. Microsomes (50-80 μg of protein) were incubated at 37 C for the specified lengths of time with 40 mM Tris-maleate buffer (pH 7.4) and either 3.0 μM $FeSO_4$ plus 250 μM NADPH or 1.0 μM $FeSO_4$ plus 500 μM ascorbate in a total volume of 0.5 ml. Peroxidation was terminated by rapid addition of 20% trichloroacetic acid (0.15 ml), 0.05 M thiobarbituric acid (0.3 ml) and 0.2% butylated hydroxytoluene (50 μl). Bovine serum albumin (0.5 mg) was added to facilitate precipitation of protein during a 10 min centrifugation; the resulting clear supernatant was removed and delivered to glass test tubes which were then tightly capped and boiled for 8 min. The amount of colored product was measured spectrophotometrically as described by Buege and Aust (4).

Lipid peroxidation was expressed in terms of nmol malondialdehyde (MDA)/mg protein or as "percent peroxidation" which is simply the percentage of maximal MDA which would be produced by complete peroxidation of the peroxidizable polyunsaturated fatty acids (PPUFA) present in the tissue fractions. PPUFA includes all of the detectable polyunsaturated fatty acids except linoleic acid (18:2), which we have demonstrated, in agreement with others (20,21), to be relatively resistant to peroxidation and is not believed to evolve malondialdehyde (22). Maximal MDA formation per mg of protein was routinely determined for each liver microsomal preparation by incubating with a sufficient amount of $FeSO_4$ and ascorbate over time until MDA formation reached a maximal value. We have previously shown that all of the liver microsomal PPUFA has reacted at this point and that the percentage of the maximal MDA formation obtained during a given incubation closely correlates with the percentage depletion of peroxidizable lipid substrate (17).

In separate experiments, the yield of MDA/mol PPUFA was found to be similar for all tissues studied. Thus, in these studies, the determination of the maximal MDA formation in lung, heart, kidney, brain and testes microsomes was obtained simply by multiplying the value for the yield of MDA/mol PPUFA from liver microsomes by the PPUFA content of the other tissues as determined by phospholipid (23) and fatty acid analysis by gas liquid chromatography (GLC) (24). The amount of

MDA produced during the experimental incubations was then expressed in terms of the percentage of the maximal MDA value and referred to as "percent peroxidation." Thus, "percent peroxidation" is equivalent to the percentage of peroxidizable polyunsaturated fatty acids reacted. Values for 100% peroxidation in terms of nmol MDA/mg protein and microsomal PPUFA contents as nmol PPUFA/mg protein are given in each figure legend (means and standard deviations). Vitamin E was measured using the spectrofluorometric technique of Taylor et. al. (16). Using this method, tissue samples are saponified and extracted yielding a hexane phase which contains partially purified vitamin E. Recovery of an internal α-tocopherol standard was 95%.

Liposomes were prepared by extracting lipids from microsomes by the Bligh and Dyer procedure (25). The chloroform solution was evaporated to dryness under N_2 gas in a glass vessel. The resulting lipid film was hydrated and ultrasonically dispersed into an aqueous suspension in a sonicating bath under N_2 gas. Where indicated, vitamin E extracted from microsomes by the Taylor et. al. procedure (16) or authentic α-tocopherol subjected to the same procedure was incorporated into liver liposomes by mixing the hexane extract with the chloroform solution of liver microsomal lipids prior to drying and dispersing.

RESULTS

Figure 1 shows the time course of lipid peroxidation in rat liver, brain, kidney, testes, heart and lung microsomes as induced by NADPH and ferrous iron. The rate of peroxidation of lung microsomes was extremely low, relative to liver, brain, kidney and testes microsomes. Heart microsomes exhibited a rate of peroxidation slightly higher than lung microsomes but still 20-fold lower than liver microsomes at the early time points. These differences were not caused by differences in peroxidizable fatty acid content since the susceptibility of the various microsomes to peroxidation did not correlate with their PPUFA content (see Fig. 1) and the data are expressed in terms of the percentage of peroxidizable lipids that had reacted.

Although the NADPH-dependent peroxidation is known to be mediated by the microsomal NADPH-cytochrome P-450 reductase (26), differences in peroxidizability could be only partially explained by tissue differences in the activity of this enzyme. Using cytochrome c as an electron acceptor, specific activities (sp act) were found to be 205, 46, 37, 25, 30 and 91 nmol cytochrome c reduced/min/mg protein for liver, brain, kidney, testes, heart and lung microsomes, respectively. These values obviously do not correlate with the pattern of microsomal peroxidation observed in Figure 1, suggesting the reductase activity is not limiting the rate of peroxidation under these conditions.

In order to more accurately assess the relative peroxidizability of the microsomes, rates of peroxidation were determined using ascorbate instead of NADPH. Ascorbate is believed to stimulate peroxidation in a manner similar to NADPH, i.e., by promoting reduction of iron (17), although the ascorbate/iron system involves a direct, rather than enzyme-catalyzed reduction of iron (27) and is therefore independent of the microsomal NADPH-cytochrome P-450 reductase activity. Results of these experiments are shown in Figure 2. Rates of ascorbate/iron-induced peroxidation were similar to those obtained with NADPH/iron (Fig. 1), except that liver microsomes peroxidized less rapidly in the ascorbate-stimulated system. This difference probably results from the much higher reductase activity of liver microsomes which may enhance the rate of NADPH-dependent peroxidation in this fraction relative to the microsomes from other

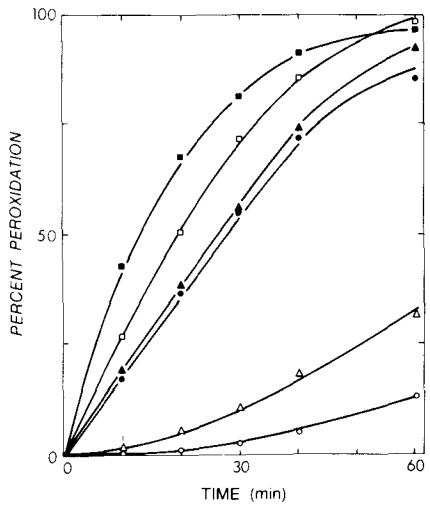

FIG. 1. Rate of NADPH/Fe^{2+}-induced lipid peroxidation in microsomes from various tissues of rat. (●) liver, (■) brain, (▲) kidney, (□) testes, (△) heart, (○) lung microsomes. Each point represents the mean value from 4 determinations. One hundred percent peroxidation is equivalent to 108 ± 11, 67 ± 15, 54 ± 9, 36 ± 4, 62 ± 14, and 92 ± 8 nmol of MDA per mg of protein for liver, brain, kidney, testes, heart, and lung microsomes, respectively. The PPUFA contents of these same microsomes were 803 ± 92, 495 ± 145, 403 ± 62, 271 ± 31, 458 ± 82, and 677 ± 62 nmol of PPUFA per mg of protein, respectively.

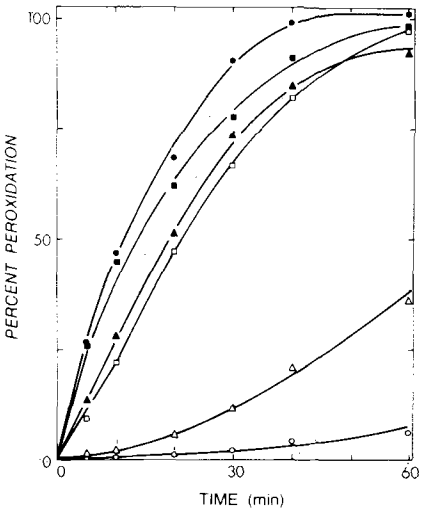

FIG. 2. Rate of ascorbate/Fe^{2+}-induced lipid peroxidation in microsomes from various tissues of rat. Symbols, values for 100 percent peroxidation, and PPUFA contents are the same as in Figure 1. Each point represents the mean value from 4 determinations.

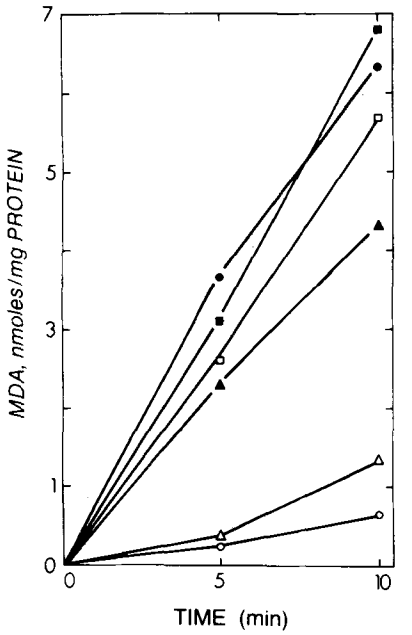

FIG. 3. Rate of Fe^{2+}-induced lipid peroxidation in cell-free homogenates of various rat tissues. Homogenates were prepared as described in Methods and incubated with 40 mM Tris-maleate (pH 7.4) and 40 μM $FeSO_4$ (Note: The homogenates contained EDTA which was present as a 30 μM concentration such that the concentration of free iron, which is effective in inducing peroxidation, was around 10 μM). Symbols are the same as in Figure 1. Points are the mean values from 2 determinations.

tissues.

In order to determine whether the rates of peroxidation in microsomes were representative of the whole tissue or organ, we performed similar measurements using tissue homogenates. As shown in Figure 3, heart and lung homogenates were peroxidized to a much lesser extent than homogenates of the other tissues studied, a result which demonstrates the uniqueness of these tissues as well as the validity of using microsomes as an indicator of whole tissue peroxidizability.

Attempts to elucidate the nature of these apparent tissue differences in susceptibility to peroxidation focused on the differences between lung and liver microsomes. Fatty acid analysis of lung and liver microsomes following incubation revealed that the polyunsaturated fatty acids of the lung microsomes were only slightly decreased under conditions which resulted in total reaction of the peroxidizable lipids of the liver microsomes (data not shown). Increasing concentrations of NADPH or ascorbate did not increase the rate of lung microsomal peroxidation. As shown in Figure 4, peroxidation of lung microsomes changed only slightly as a function of increasing iron concentration, whereas liver microsomal peroxidation increased dramatically. In addition, varying the incubation pH did not augment lung microsomal peroxidation relative to liver microsomal peroxidation (data not shown). Thus, the lack of peroxidation of lung microsomes apparently is not attributable to limiting concentrations of iron, NADPH or ascorbate, or to suboptimal

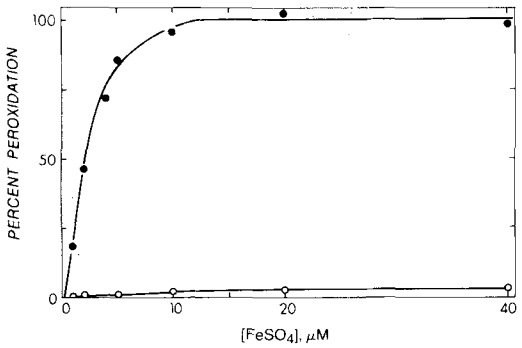

FIG. 4. Lipid peroxidation in rat liver and lung microsomes as a function of ferrous iron concentration. Liver (●) and lung (○) microsomes were incubated for 15 min with 250 μM NADPH and $FeSO_4$ as indicated. Points are the mean values from 4 determinations. 100% peroxidation is equivalent to 85 ± 8 and 70 ± 2 nmol MDA/mg protein for liver and lung microsomes, respectively. The PPUFA contents of these same microsomes were 633 ± 4 and 520 ± 59 nmol PPUFA/mg protein, respectively.

TABLE I

Vitamin E Content of Microsomes from Various Tissues of Rat[a]

Source of microsomes	μg Vitamin E/mg protein	μg Vitamin E/mg PPUFA[b]
Lung	0.77 ± 0.01	4.3 ± 0.4
Heart	0.26 ± 0.05	1.9 ± 0.6
Liver	0.16 ± 0.03	0.73 ± 0.01
Kidney	0.09 ± 0.02	0.69 ± 0.14
Testes	0.10 ± 0.04	0.67 ± 0.07
Brain	0.09 ± 0.04	0.50 ± 0.15

[a]Values are the means ± standard deviations from 4 experiments. The values for μg vitamin E/mg PPUFA can be converted to mmol vitamin E/mol PPUFA by multiplying by 0.76.

[b]PPUFA refers to peroxidizable polyunsaturated fatty acids (18:3, 20:3, 20:4, 20:5, 22:4, 22:5, and 22:6).

pH.

One possible explanation for the different rates of peroxidation in the different tissues is that the microsomes contain different amounts of the biological antioxidant, vitamin E. In order to test this hypothesis, we measured levels of vitamin E in the different microsomes: the results are shown in Table I. Vitamin E was quantitated on a per mg protein basis as well as per mg PPUFA; the PPUFA ratio probably more accurately represents the antioxidant capacity of the tissue. For lung microsomes, the ratio of vitamin E-to-PPUFA was 6-8 times higher than in liver, kidney, testes and brain microsomes, which is consistent with the much slower rate of peroxidation in lung microsomes (Fig. 3). Heart microsomes, which peroxidized faster than lung but slower than liver, kidney, testes or brain microsomes contained an intermediate amount of vitamin E/mg PPUFA.

We also studied rates of peroxidation and vitamin E content in microsomes from different species. As shown in Figure 5, peroxidizability of rabbit microsomes from various tissues was similar to rat microsomes (Fig. 2) and again there was an inverse correlation with the ratio of vitamin E-to-PPUFA, shown in Figure 6. Some variation among species in these parameters was observed, however. Figure 7 summarizes the data on lung microsomes from rats, rabbits, mice and humans. Rabbit lung microsomes exhibited the highest vitamin E/PPUFA ratio, and practically no lipid peroxidation was detectable after a 30 min incubation. In contrast, peroxidation was by far the highest in mouse lung microsomes and the vitamin E/PPUFA ratio was nearly 5 times less than in rabbit lung microsomes. Peroxidizability and vitamin E content of human lung microsomes was most similar to rat lung microsomes. These correlations suggest microsomal susceptibility to lipid peroxidation is determined primarily by the ratio of vitamin E-to-PPUFA present in the microsomal membranes. The relative resistance of lung microsomes to peroxidation, then, appears to result from the high levels of vitamin E.

In order to further substantiate this hypothesis, we measured the rate of ascorbate/iron-induced peroxidation in liposomes prepared from extracted microsomal lipid. Figure 8a shows that liver liposomes were over 50%

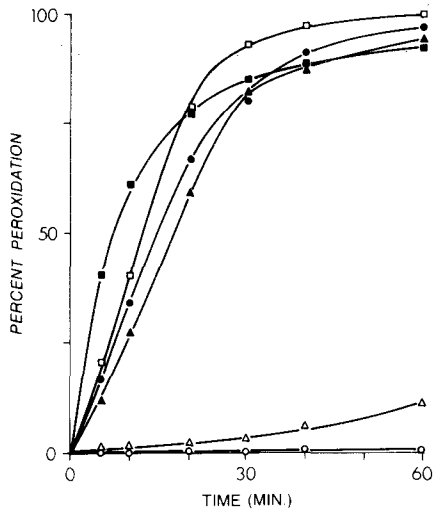

FIG. 5. Rate of ascorbate/Fe^{2+}-induced lipid peroxidation in microsomes from various tissues of rabbit. Symbols as in Figure 1. Points are the mean values from 4 determinations. 100% peroxidation is equivalent to 81 ± 11, 109 ± 14, 62 ± 22, 57 ± 5, 37 ± 4, and 82 ± 9 nmol MDA/mg protein for liver, brain, kidney, testes, heart and lung microsomes, respectively. The PPUFA contents of these same microsomes were 561 ± 25, 752 ± 105, 423 ± 88, 394 ± 12, 251 ± 49, and 562 ± 37 nmol PPUFA/mg protein, respectively.

peroxidized in 30 min whereas lung liposomes under the same conditions were almost completely resistant to peroxidation. As shown in Figure 8b, incorporation of the vitamin E extracted from an equivalent amount of lung microsomes (or an amount of authentic vitamin E equal to that found in the lung microsomes) into liver lipid produced liver liposomes which were also resistant to peroxidation. In contrast, vitamin E extracted from an equivalent amount of liver microsomes and added to liver lipid (essentially doubling the vitamin E content of the liver liposomes) had only a modest effect on the rate of peroxidation relative to control liver liposomes. These findings demonstrate that the 6-fold greater amount of vitamin E in lung compared to liver (Table I) is sufficient to account for the observed resistance of lung liposomes and microsomes to peroxidation.

DISCUSSION

The vitamin E content of various tissues is a somewhat ambiguous concept depending upon the choice of denominators. Previous studies of tissue vitamin E content expressed as μg/g tissue (28,29) did not reveal some of the striking differences among the tissues studied here. Since vitamin E is probably located in cell membranes, it seems most useful to quantitate its presence in tissues as a function of some parameter of the tissue membrane content. Thus, we have chosen to express vitamin E

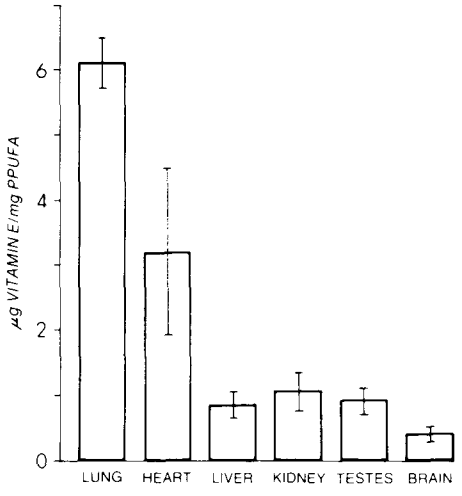

FIG. 6. Vitamin E content of microsomes from various tissues of rabbit. Values shown are means and standard deviations from 3 determinations. Microsomal PPUFA contents were the same as in Figure 5. The values for μg vitamin E/mg PPUFA can be converted to mmol vitamin E/mol PPUFA by multiplying by 0.76.

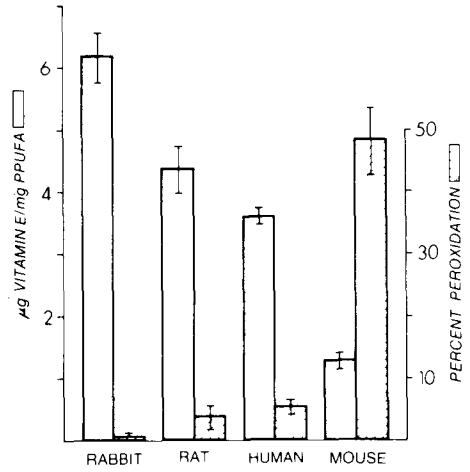

FIG. 7. Lipid peroxidation and vitamin E content of lung microsomes from different species. Lipid peroxidation was determined following a 30 min incubation of microsomes with the ascorbate/Fe^{2+} system. Other procedures are described in Methods. Values shown are means and standard deviations from 2-4 determinations. 100% peroxidation is equivalent to 82 ± 9, 92 ± 8, 82, and 81 ± 13 nmol MDA/mg protein for rabbit, rat, human and mouse lung microsomes, respectively. The PPUFA contents of these same microsomes were 562 ± 37, 677 ± 62, 533, and 536 ± 64 nmol PPUFA/mg protein, respectively. The values of μg vitamin E/mg PPUFA can be converted to mmol vitamin E/mol PPUFA by multiplying by 0.76.

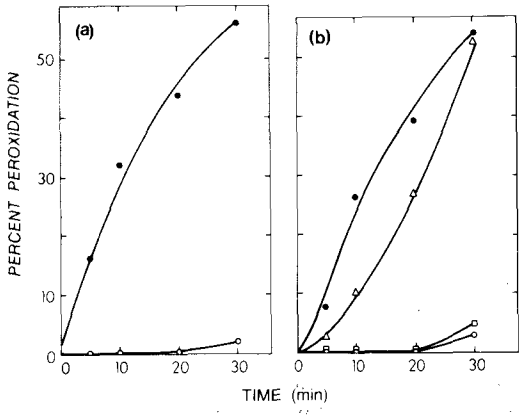

FIG. 8. Peroxidation of liver and lung liposomes by ascorbate/iron. Liposomes were prepared or vitamin E extracted from aliquots of lung or liver microsomes containing equal amounts of PPUFA. (a) Liver liposomes (●), lung liposomes (○); (b) liver liposomes with incorporated vitamin E from lung microsomes (○), liver liposomes with incorporated authentic vitamin E equal to that in lung microsomes (□), liver liposomes with incorporated vitamin E from an equal additional sample of liver microsomes (△), liver liposomes plus vitamin E extract of a water blank (●). Each point is the mean value from 3 determinations.

content on the basis of PPUFA of the microsomes as well as per mg microsomal protein. The values obtained by this method correlate with microsomal susceptibility to lipid peroxidation and can account for the high resistance of lung and heart microsomes to peroxidation. Consistent with our findings, Taylor et al. (16) observed 7-fold and 5-fold higher amounts of vitamin E/mg microsomal protein in lung and heart, respectively, compared to liver microsomes. The Evarts and Bieri data (30) for whole tissue of rats fed soybean oil agree well with our microsomal ratios of vitamin E-to-PPUFA in heart liver and kidney. Whole lungs showed a 30% lower ratio compared to our microsomal data, suggesting that, compared to whole tissue, lung microsomes are enriched in vitamin E relative to PPUFA. Whole testes, in contrast, showed a 73% higher vitamin E/PPUFA ratio than our microsomal data, suggesting a nonmicrosomal enrichment of vitamin E in that tissue.

The presence of vitamin E in lung and heart microsomes at levels sufficient to almost totally prevent peroxidation under conditions which resulted in complete peroxidation of microsomes from other tissues raises serious questions regarding lipid peroxidation as a mechanism of pathological or toxocological damage in lung or heart. Apparently, peroxidation of lung or heart microsomes could only occur after depletion of their relatively higher content of vitamin E.

Vitamin E has long been recognized as a membrane-soluble antioxidant (9). Its importance as a biological protective agent relative to soluble antioxidant factors such as glutathione peroxidase or superoxide dismutase has not been established. Our results show that, in the absence of soluble factors, the susceptibility to peroxidation of microsomes from several tissues and species correlates with the microsomal vitamin E content. Since lung and heart microsomes are highly resistant to lipid peroxidation in the absence of soluble factors, the need for soluble antioxidants in these tissues is not apparent with respect to protection of microsomal membrane lipids. The interesting peroxidizability of whole tissue homogenates (Fig. 3) also reflected microsomal vitamin E content. Lung and heart homogenates underwent very little peroxidation under conditions which produced several-fold greater peroxidation in homogenates of brain, kidney, liver and testes. Thus, it is clear that in the tissues containing lesser amounts of vitamin E, there are no soluble factors which are effective under these conditions in protecting against lipid peroxidation to the same extent that vitamin E protects lung and heart. It is possible that glutathione peroxidase, which is about twice as active in liver as in lung or heart (31), may not function effectively under these conditions because of possible depletion of reducing potential in the homogenate. Soluble superoxide dismutase, however, is over 6 times more active in liver than in lung (32) and might be expected to function in the homogenates, since no depletable cofactors are required. Thus, the amount of protection against lipid peroxidation afforded by these 2 enzymes is not apparent from our data.

The protection of lung and heart against peroxidation by a high content of membranous vitamin E is teleologically sound considering the relatively high oxygen tension in the lung and the exposure of heart to freshly oxygenated blood from the lungs. The mechanism of vitamin E uptake and retention by tissues is unknown and, thus, the biochemical basis for high levels of membranous vitamin E in lung and heart remain to be elucidated. The cellular distribution of the lung vitamin E also remains an intriguing area for future investigations, since the lung contains a number of different cell types (33).

ACKNOWLEDGMENTS

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Skin Surface Lipids of the Horse

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ABSTRACT

Skin surface lipids from the sides of male and female horses *(Equus caballus)* were collected in acetone and analyzed by thin layer chromatography and gas liquid chromatography. The sole components in both sexes were cholesterol, cholesteryl esters and the lactones of 32-, 32- and 36-carbon ω-hydroxy acids, each including a methyl group in the n-1 position. Most of the lactones were monounsaturated (either n-8 or n-10), but small amounts of saturated and dienoic species were present. A pooled sample of the skin surface lipids contained 14% cholesterol, 38% cholesteryl esters and 48% lactones.

INTRODUCTION

Previous studies have shown that the skin surface lipids of mammals differ profoundly among species, both in the classes of lipids present and in the structures of the aliphatic and alicyclic moieties (1-4). So far, however, such studies have not included more than one species from any genus, and we have been curious whether similarities in surface lipid composition might be observed in closely related species. In order to study this question, we have chosen the genus *Equus* because it provides a favorable situation in which to find such similarities, since the constituent species are so similar in morphology, diet and lifestyle. A previous survey by thin layer chromatography (TLC) (1) had shown that the surface lipids of the domestic horse *(Equus caballus)* contained a major constituent which migrates like a wax diester, together with some sterol esters and free sterols. We have now been able to demonstrate that the apparent diester fraction actually consists of the lactones of long chain, methyl-branched ω-hydroxy acids. The fatty acids of the sterol esters include similar structures without a hydroxyl substituent. The free sterols and those from the sterol esters consist almost exclusively of cholesterol.

MATERIALS AND METHODS

Collection of Surface Lipids

About 100 ml of acetone was poured onto the side of each horse about 30 cm behind the foreleg and the solvent was then recovered by scraping upward with a 1 ℓ beaker pressed to the skin. The beaker had been prepared for this purpose by cutting off half of the rim at an angle of 45° and then fire polishing the cut surface. With this device, recoveries of solvent were at least 90% efficient. The resulting solutions were filtered through a sintered glass funnel and then evaporated on a rotary evaporator, yielding 50-100 mg of lipid per animal.

Preliminary TLC Analysis

Analysis of aliquots of the collected lipids on silica gel using toluene as developing solvent or a triple development with hexane, then toluene and then hexane/ether/acetic acid (70:30:1) indicated the overall composition of the collected lipids was virtually identical for 1 male and 2 female horses. Quantitative TLC analysis (5) of the pooled material showed that the lipids, identified as described below, consisted of 48% lactones, 38% cholesteryl esters and 14% cholesterol. Preparative TLC of the mixture, using toluene development, separated the 3 fractions, which were examined as follows.

Lactones. The fraction corresponding in TLC mobility to wax diesters was hydrolyzed with 5% KOH in methanol/water/toluene (95:5:50) at 50 C and the recovered lipid produced a single spot on TLC migrating in the same way as long chain ω-hydroxy acids obtained from carnauba wax (6). The following diagnostic procedures, in which the carnauba wax hydroxy acids were used as model compounds, led to the conclusion that the major fraction in the horse surface lipids consisted of the lactones of C_{30}-C_{38} ω-hydroxy acids.

(a) After methylation with BCl_3/MeOH, the hydroxy acids from the horse lactones and from carnauba wax increased similarly in TLC mobility.

(b) The methyl esters of the hydroxy acids from both sources were oxidized with chromic acid in acetone (7) to form the half esters of dicarboxylic acids, resulting in a further increase in TLC mobility. These compounds were converted to dimethyl esters with BCl_3/MeOH, producing a still greater increase in TLC migration.

(c) The carnauba hydroxy acids were cyclized to lactones by refluxing them in benzene (20 mg/ℓ) with *p*-toluenesulfonic acid (20 mg/ℓ) as catalyst (8). Similar treatment of the hydroxy acids from the horse produced a

material having the same TLC mobility as the original fraction from the surface lipid and the same as the lactones derived from carnauba wax.

(d) A large sample of the horse lactones was fractionated on silica gel/$AgNO_3$ (toluene solvent) into saturated (16%), monoenoic (78%) and dienoic (6%) fractions, and these were analyzed by gas liquid chromatography (GLC) on an OV17 column at 340 C. The proton magnetic resonance (PMR) spectrum of each of the argentation fractions showed a 1:1 doublet (0.92 ppm, J = 6.6 cps, equivalent to 3 protons) at the position characteristic of methyl group absorption, as well as a 1:1 doublet (3.95 ppm, J = 6.1 cps, 2 protons) at a position indicative of protons on a carbon atom attached to an alkyl oxygen (Fig. 1). This indicated a structure in which the carbon atom bearing the alkyl oxygen was adjacent to the same carbon atom as the methyl group. Both the monoenoic and dienoic lactone fractions showed the 1:2:1 triplet absorption of ethylenic protons (5.41 ppm, J = 4.5 cps) equivalent to 2 and 4 protons, respectively. However, the dienoic lactones showed no absorption near 2.8 ppm expected for a methylene group located between 2 ethylenic bonds in methylene-interrupted diunsaturation.

(e) To determine the position of unsaturation in the monoenoic lactones, a sample of this fraction was first separated into 32-, 34- and 36-carbon ring fractions by high performance liquid chromatography (HPLC) on μBondapak C_{18} (Waters Associates, Milford, MA) using 10% $CHCl_3$ in methanol as eluting solvent. A 5 mg aliquot of each fraction was then oxidized by the von Rudloff method (9) and the products were recovered in chloroform. The solvent was evaporated and the residue in each case was saponified by heating at 50 C for 1 hr in 1.5 ml of 5% KOH in methanol/water/toluene (95:5:50). The hydrolysis products were then directly methylated by addition of BCl_3/MeOH (2 ml) and after 10 min were recovered by addition of water and extraction into chloroform. The products then consisted of the dimethyl esters of dicarboxylic acids (from the fragment of the lactones between the double bond and the carboxyl group) together with the methyl esters of short chain hydroxy acids (derived from the sections between the double bond and the hydroxyl group), as shown in Figure 2. These 2 groups of products were separated by preparative TLC on silica gel and the dimethyl esters were analyzed by GLC. Reference dimethyl esters were prepared by oxidation of the carnauba hydroxy acids with chromic acid in acetone (7) followed by methyl-

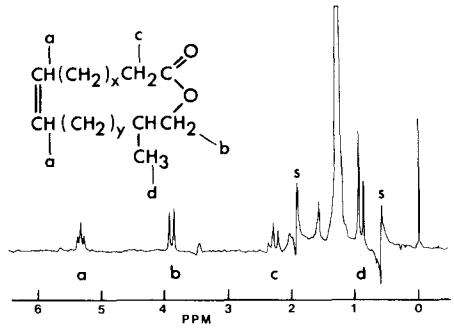

FIG. 1. PMR spectrum of the monoenoic lactones from horse skin surface lipids, obtained at 90 MHz in deuterochloroform using a Bruker HX-90E instrument modified for high power pulsed Fourier transform data collection. The peaks labeled "s" are side bands of the methylene absorption.

ation with BCl_3/MeOH. Each lactone fraction yielded only 2 diester fragments, which were straight chained and resulted from the loss of either 9 or 11 carbon atoms from the original lactones. The hydroxy ester fragments were oxidized in CrO_3/acetone and the resulting diacid half esters were methylated and analyzed by GLC. The analysis indicated each of the different lactone ring sizes yielded the same 2 products, namely the methyl-substituted 8- and 10-carbon dicarboxylic acids. This showed that the monoenoic lactones in each ring size contained either an n-8 or n-10 double bond; the 2 positional isomers were present in approximately equal amounts. The quantitative analysis of the lactone structures is shown in Table I.

(f) Confirmation of the proposed lactone structures was obtained by mass spectrometry, using a Perkin Elmer/Hitachi Model 6E instrument. Each lactone prepared from the carnauba wax hydroxy acids produced a molecular ion and an M-18 peak of about half the intensity of

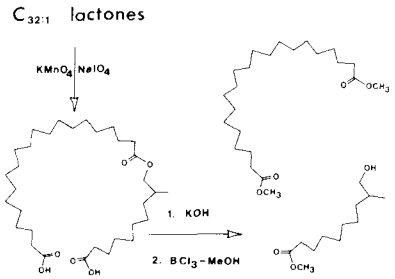

FIG. 2. The reaction scheme used in determining the positions of unsaturation in the pure chain length fractions from the monoenoic lactones of horse skin surface lipid.

TABLE I

Composition (wt %) of the Giant-ringed Lactones from Horse Skin Surface Lipids

Total carbon atoms	Saturated	Monoenoic[a]	Dienoic
30	0.5	0.3	-
31	0.6	0.2	-
32	7.8	16.4	0.3
33	1.4	2.2	0.1
34	4.7	47.3	2.3
35	0.4	1.8	0.6
36	0.7	9.4	2.3
37	-	-	-
38	-	-	0.3
Totals	16.1	77.6	5.9

[a]The ratios of the n-8 and n-10 positional isomers in the C_{32}, C_{34} and C_{36} monoenoic acids were 53:47, 55:45 and 45:55, respectively.

the M^+ ion. These were the most abundant of the high-mass ions, as might be expected for molecules which must fragment twice to produce ions of lower mass. The lactones from the horse surface lipids produced molecular ions of M/e 476, 504 and 532, which is appropriate for the suggested structures of the C_{32}, C_{34} and C_{36} monoenoic lactones. Each was accompanied by an M-18 ion, but these were of somewhat lower abundance than the M-14, M-15 and M-16 ions, which presumably resulted from a loss of the methyl side chain with or without the gain or loss of a hydrogen.

Cholesterol and cholesteryl esters. The free sterol and that obtained upon hydrolysis of the sterol esters each gave a single spot on TLC, migrating in the same way as authentic cholesterol and showing the same color changes upon charring with sulfuric acid. GLC analysis showed essentially a single peak with the same retention time as cholesterol for both sterol fractions.

The fatty acids produced by hydrolysis of the cholesteryl esters were converted directly to methyl esters by addition of BCl_3/MeOH to the saponification mixture. The methyl esters and the liberated sterol were then separated by preparative TLC. GLC of the methyl esters showed the fatty acids had a very wide distribution of chain length, from C_{16} to C_{38}, and contained both saturated and unsaturated components. The presence of unsaturation was confirmed by repeating the gas chromatography after the esters had been hydrogenated with Pt/H_2 in ether. The gas chromatograms also revealed that almost all of the fatty acids, both saturated and unsaturated, were branched chain compounds, and this was confirmed by examining the PMR spectrum (Fig. 3), which showed a 1:1 doublet at 0.86 ppm (J = 6.1 cps) equivalent to 6 protons, indicating the *gem*-dimethyl groups of isobranched acids. The quantitative analysis of the cholesterol ester fatty acids is shown in Table II.

When the mixture of methyl esters was subjected to TLC on silica gel/$AgNO_3$ for separation by degree of unsaturation, 2 broad bands were produced, the more mobile of which contained the saturated esters together with the longer chain (C_{24}-C_{36}) monounsaturated esters. The less mobile band was found to contain the C_{18}-C_{24} monoenes as well as the C_{32}-C_{38} dienes. These incomplete resolutions resulted from the very wide range of chain lengths in the monounsaturated acids. When the

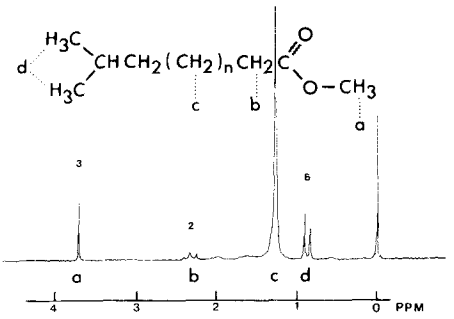

FIG. 3. PMR spectrum of the "saturated" fraction of methyl esters of the fatty acids from the cholesteryl esters of horse skin surface lipids. The spectrum was obtained as described for Figure 1, except that the sample tube was plugged and spun at high speed to minimize the effect of side bands.

TABLE II

Composition (wt %) of the Fatty Acids from the Cholesterol Esters of Horse Skin Surface Lipids

Total carbon atoms	Saturated		Monoenoic	Dienoic
	Straight	Branched		
16	0.7	5.6		
17	-	0.5		
18	0.5	4.9	1.6	
19	-	0.5	0.2	
20	0.4	12.9	8.2	
21	-	0.7	0.2	
22	0.3	4.7	3.8	
23	-	0.3	-	
24	0.3	2.7	0.9	
25	-	0.3	-	
26	0.2	3.4	1.1	
27		0.2	-	
28		2.5	0.7	
29		0.1	-	
30		2.3	0.9	
31		-	-	
32		1.8	5.9	
33		-	-	
34		1.0	15.4	0.8
35			0.2	-
36			5.9	1.4
37			-	-
38			1.7	0.5
Totals	2.6	44.7	46.7	2.7

less mobile band was oxidized with periodate-permanganate (9) and the products examined by GLC using the Downing and Greene method (10), the monocarboxylic acid fragments were found to consist of almost equal quantities of C_9 and C_{11} branched chain acids, whereas the dicarboxylic fragments were predominantly the C_9 and C_{11} acids, with small amounts of the C_{12} and C_{13} compounds. Thus, the double bonds in the C_{18}-C_{24} fatty acids from the cholesteryl esters were confined to positions which would have been produced by $\Delta 9$ desaturation of C_{18} and C_{20} saturated fatty acids followed by some chain extension. Similar oxidative determination of double bond positions in the longer chain monoenes contained in the more mobile band on argentation-TLC gave equal amounts of the same branched chain C_9 and C_{11} monocarboxylic acids, showing that the double bond positions were derived simply by further chain extension from the shorter monounsaturated acids.

DISCUSSION

Giant-ringed lactones of the type observed in this study apparently have not been found previously as natural products, although ω-hydroxy acids of similar molecular weight (MW) have been found in the surface lipids of sheep (11) and are widespread in the surface lipids of plants (12,13). The ω-hydroxy acids of wool wax include some branched chain compounds, whereas those of the plant lipids are entirely straight chained and thus are not a possible source of the lactones observed on the horses. It has not been established in what form the ω-hydroxy acids exist in wool wax or in the soluble lipids of plant surfaces, so that the occurrence of lactones cannot be excluded. The lactone of a shorter chain ω-hydroxy acid, 16-hydroxy-7E-hexadecenoic acid (ambrettolide), is present in musk seed oil and is responsible for its odor.

We presume the lactones and the cholesteryl esters are produced by the sebaceous glands in the skin of the horse. The probable pathway of biosynthesis of the lactones is indicated by the positions of the ethylenic bonds in the monoenoic series, which could be produced by $\Delta 9$ desaturation of C_{18} and C_{20} fatty acids, followed by chain elongation, hydroxylation and, finally, cyclization. The second double bond in the dienoic lactones apparently is not introduced in the usual methylene-interrupted pattern, and may result from a second $\Delta 9$ desaturation after chain extension.

The fatty acids found esterified with cholesterol are remarkable for the very small proportion of straight chain components and for the very wide range of chain lengths. These fatty acids also are similar in distribution (in the C_{32}-C_{36} range) to the lactones, with which they could share a common origin or a precursor/product relationship.

It is generally inferred that one of the principal functions of the sebaceous lipids is to provide a water-resistant protection for the pelt, but this does not explain why it has been advantageous for different species to evolve diverse sebum compositions; speculation thus has continued on other possible functions for the sebaceous lipids. Lactones from the horse (equolides) have a cyclic structure, a methyl branch near the oxygen function and a location of the ethylenic bond inviting comparison with the large-ringed ketones, muscone and civetone, which perform pheromonal functions in the musk deer and the civet cat, respectively. If the equolides have such a function for the horse, it presumably is nonsexual, since the compounds are found in similar quantities in both sexes.

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Dietary Arachidonic Acid Reduces Fatty Liver, Increases Diet Consumption and Weight Gain in Ethanol-fed Rats[1]

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ABSTRACT

We fed young male Sprague-Dawley rats for 4 wk ad libitum liquid diets containing 34% of the calories as ethanol and 35% as fat with (AA+) and without (AA-) arachidonic acid (20:4). Additional rats in the control groups were fed similar diets made isocaloric with dextrose with (CA+) and without (CA-) 20:4. The liver triglyceride (TG) content of rats in the AA+ group was reduced ca. 3-fold over that of rats in the AA-group. The diet consumption and body wts of rats in the AA+ group were significantly greater than those of rats fed alcohol without the 20:4 supplement (AA-). Also livers from rats in the AA+ group were as large as those from rats in control groups (CA+, CA-) and ca. twice as large as those from rats in the AA-group. The fatty acid composition of liver TG in rats fed the alcohol diet was similar to that of dietary fat. Levels of 20:4 and docosatetraenoic acid (22:4) in liver TG fatty acids from rats fed diets without arachidonate (AA-, CA-) were low (trace to 1.6%). After ingestion of arachidonic acid, 20:4 increased to ca. 10% and 22:4 to ca. 5%. The content of liver phospholipids was higher in livers of rats fed ethanol (AA-) than in those of controls (CA-).

INTRODUCTION

Prolonged alcohol consumption leads to fatty liver—an accumulation of triglycerides in hepatic tissue. The triglycerides that contribute to fatty liver could originate from 1 or more of 3 sources: diet, fatty acids released from adipose tissue and enhanced fatty acid synthesis in the liver. It has been concluded that lipogenesis is the major cause of hepatic accumulation of triglycerides in studies where the fat content of the alcohol-containing diet was low (1-3). However, when an adequate amount of fat is fed, increased hepatic lipogenesis is believed to be of minor importance in the development of alcoholic fatty liver (4).

When a diet is fed which causes the ratio of linoleic acid (18:2) to arachidonic acid (20:4) to increase in liver total lipids, hepatic lipogenesis is stimulated; when this diet is supplemented with arachidonic acid, hepatic lipogenesis is reduced (5). Ethanol feeding also causes an increased 18:2/20:4 ratio in lipids from liver as well as many other tissues (6-12). Therefore, it may be expected that hepatic lipogenesis would be stimualted in alcohol-fed animals. However, the role of fatty acid synthesis in vivo in elevating hepatic triglycerides has been controversal (3,13,14). Regardless of the amount of lipogenesis, dietary 20:4 could be useful in reducing liver fatty acid synthesis in alcohol-fed animals.

Arachidonic acid is a naturally occurring component of phospholipids. Phospholipids are components of lipoproteins, the vehicles for the removal of triglycerides from liver. Therefore, reduced levels of 20:4 could decrease the mobilization of fat from liver and hence contribute further to hepatic fat accumulation.

In this study, we fed rats an ethanol diet with arachidonic acid to determine whether dietary 20:4 influences the accumulation of liver triglycerides. Since the effects of feeding this acid to ethanol-fed animals have been unknown, we maintained rats with free access to diets to observe how 20:4 also influences diet consumption and weight gain. This study will provide a basis from which to conduct further studies on the effects of arachidonic acid on alcoholic fatty liver.

MATERIALS AND METHODS

Animals and Diets

Twelve male Sprague-Dawley rats weighing 200 g were obtained from Charles River, Inc. (Willmington, MA). Animals were fed the Wayne Lab Blox diet (Allied Mills, Chicago) ad libitum for 1 wk. Animals were divided into 4 groups of 3 each, were housed in stainless steel cages and fed through glass Richter tubes. Each group was fed ad libitum for 4 wk one of the following isocaloric Lieber/DeCarli liquid rat diets (Bio-Mix #711, Bio-Serv., Inc., Frenchtown, NJ): (a) control diet (CA-), (b) ethanol diet (AA-), (c) control diet with 7 weight % of the fat as arachidonic acid (CA+), and (d) ethanol diet with 7 wt % of the fat as arachidonic acid (AA+). Arachidonic acid, as obtained from the manufacturer (Nu-Chek-Prep, Inc., Elysian, MN), was analyzed by gas liquid chromatography (GLC) and determined to be

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70% pure. The fatty acid composition of the arachidonic acid was as follows: 16:1, 3.3%; 18:1, 1.3%; 18:2, 17.4%; 18:3, 1.4%; 20:3, 5.5%; 20:4, 69.8%, 22:4, 1.3% (fatty acids are designated as the number of carbons:the number of double bonds). Arachidonic acid was added to prepare the CA+ and AA+ diets such that 7 wt % of the dietary fat was 20:4. Butylated hydroxytoluene (BHT, 0.02%) was added to the fat used to prepare the diets. Diets were mixed according to supplier's instructions except for the addition of 20:4. Diets were kept at 5 C under nitrogen and used within 72 hr. Fatty acid analysis of the diets indicated there was no detectable loss of unsaturated fatty acids during either storage or feeding. Rats were fed fresh diets every morning. Diet consumption was recorded daily for each animal. Rat body weights were determined twice/week.

Quantitation of Liver Triglycerides and Phospholipids

After 4 wk of feeding, rats were anesthetized by an intraperitoneal (ip) injection of sodium pentobarbital (50 mg/ml/300 g rat). Livers were removed, rinsed in ice cold saline, blotted and weighed. Visible pieces of mesentery tissue were removed and discarded before weighing. Small portions of liver (ca. 0.5 g) were cut from the central portion of the left lobe and weighed. These sections were homogenized in ice cold saline and lipids were extracted and washed as described by Folch et al. (15). Lipid extracts were protected from the oxidation of unsaturated fatty acids by adding BHT (0.02%) and surrounding them with a nitrogen atmosphere. An aliquot of the lipid extract corresponding to that from 25 mg of liver tissue was separated into lipid classes by silica gel thin layer chromatography (TLC) (16). The gel from regions of the plates corresponding to the triglyceride and phospholipid fractions were scraped and placed in screw-capped test tubes. Methyl esters were prepared without extracting the lipids by leaving the gels in 2.0% sulfuric acid in methyl alcohol for 16 hr at 70 C; methyl esters were then extracted and purified by TLC (17). Methyl pentadecanoic acid (13 μg) was added to the methyl esters as an internal standard. The esters were separated by gas liquid chromatography (GLC) using a Varian aerograph (Model 2740) equipped with a flame ionization detector and 6' x 1/8" stainless steel column packed with 5% diethylene glycol succinate, on H/P Chromosorb G. Percentages of various fatty acid methyl esters and their quantities were calcd using the chromatographic data system (Varian CDS-111). The retention times and relative response factors were determined using quantitative mixtures prepared from authentic fatty acid methyl esters obtained from Supelco (Bellefonte, PA) and Applied Science (State College, PA). Fatty acid composition of total liver lipids were determined as already described.

RESULTS

Diet Composition

The composition of each of the diets used are shown in Table 1. The composition with respect to protein, fat, salt and vitamins was the same in various diets used in this study. The content of carbohydrate was the same except that in the alcohol diets, some dextrose was replaced with an isocaloric amount of ethanol.

The fatty acid composition of the fat used for the preparation of these diets is shown in

TABLE I

Composition of Diets[a]

Diet[b]	Protein[c]	Ethanol	Carbohydrate[d]	Fat[e]	Salt	Vitamins
			g/l			
CA-	42.2	---	114.04	39.6	10.0	5.0
CA+	42.2	---	114.04	39.6	10.0	5.0
AA-	42.2	50.0	24.4	39.6	10.0	5.0
AA+	42.2	50.0	24.4	39.6	10.0	5.0

[a]Bio-Mix #711, Bio-Serv, Inc., Frenchtown, NJ.

[b]Diets used were control (CA-), control plus arachidonic acid (CA+), alcohol (AA-) and alcohol plus arachidonic acid (AA+).

[c]41.4 g vitamin-free casein, 0.5 g *L*-cystine, 0.3 g *DL*-methionine.

[d]Maltose-dextrins.

[e]See Table II for fatty acid composition.

TABLE II

Fatty Acid Composition of Diets[a]

Diet[b]	16:0[c]	16:1	18:0	18:1	18:2	20:4
			(%)			
CA-,AA-	10.1	0.9	2.9	60.9	25.3	---
CA+,AA+	9.0	0.9	2.7	56.4	23.9	7.1

[a]Values given indicate the percentage of total fatty acids and are mean values from duplicate determinations with each dietary fat.

[b]See Table I for diet abbreviations.

[c]Abbreviations for fatty acids indicate the number of carbon atoms:number of double bonds in the acid.

Table II. The major difference between the 2 fat mixtures is that one contained 7.1% 20:4 (CA+, AA+) whereas the other did not contain any detectable amount of this acid.

Diet Consumption

Figure 1 shows the average diet consumption of rats in each of the dietary groups. Within 3 days, all rats achieved a stable rate of food intake although animals fed either of the control diets (CA-, CA+), ate more than twice as much as those fed either of the ethanol diets (AA-, AA+) (Fig. 1). After 12 days of feeding, rats fed the control diets consumed a constant volume of food per day (ca. 100 ml) whereas those fed ethanol with arachidonic acid (AA+) began eating more than rats fed ethanol without arachidonic acid (AA-). Rats in the AA+ group continued to increase their daily food intake. At the end of the experiment (Fig. 1), they consumed nearly as much as controls (90 ml compared to 100 ml). However, after the first wk, rats on the ethanol diet (AA-) did not significantly increase their consumption.

Body and Liver Weights

Before ingesting their prescribed diets, rats weighed ca. 245 g (Fig. 2). Rats fed either of the control diets (CA- and CA+) gained ca. 150 g durng the 28 days whereas those fed the ethanol diet in the absence of 20:4 (AA-) gained only 20 g in the same period. However, when rats were fed alcohol with arachidonic acid (AA+), they gained ca. 80 g during the 4 wk interval.

For the first 2 wk, rats in the AA+ group maintained the same weight as those in the AA- group (Fig. 2). Subsequently, they began consuming more diet (Fig. 1) and increased their body weights at a rate of ca. 4 g/day, which is similar to that of rats fed the control diets (Fig. 2).

The weights of the rats at the end of 4 wk reflected their food intake (Fig. 3a). Rats fed either of the control diets (CA+, CA-) weighed considerably more (ca. 400 g) than those fed the alcohol diet without the 20:4 supplement (AA-, 276 g). When rats consumed the alcohol diet containing 20:4, their body weights increased significantly (326 g, Fig. 3a).

The weights of livers from rats in the AA+, CA+ and CA- groups were found to be similar whereas rats in the AA- group had livers which were considerably lighter (Fig. 3b). Rats in groups CA-, CA+ and AA+ consumed more diet

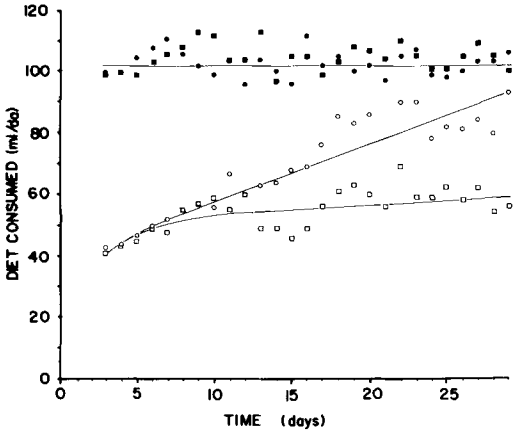

FIG. 1. Diet consumption. The figure indicates the volume of diet consumed daily by rats fed control diets with (CA+, •) and without arachidonate (CA-, ▪) as well as alcohol diets with (AA+, ○) and without (AA-, □) arachidonic acid. Values are means of measurements taken from 3 animals in each dietary group.

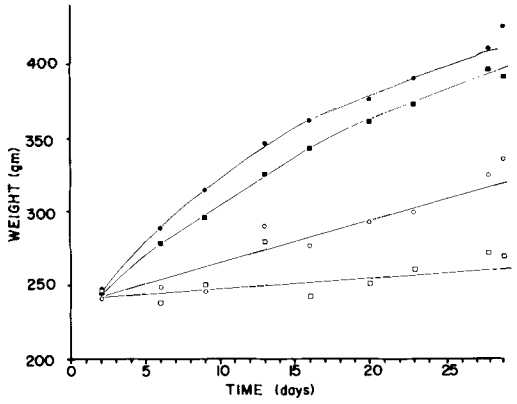

FIG. 2. Weights of rats fed the various diets. Rats were fed control diets with (CA+, •) and without (CA-, ▪) arachidonic acid as well as diets containing alcohol with (AA+, ○) and without arachidonate (AA-, □). Values are means of measurements taken from 3 animals in each dietary group.

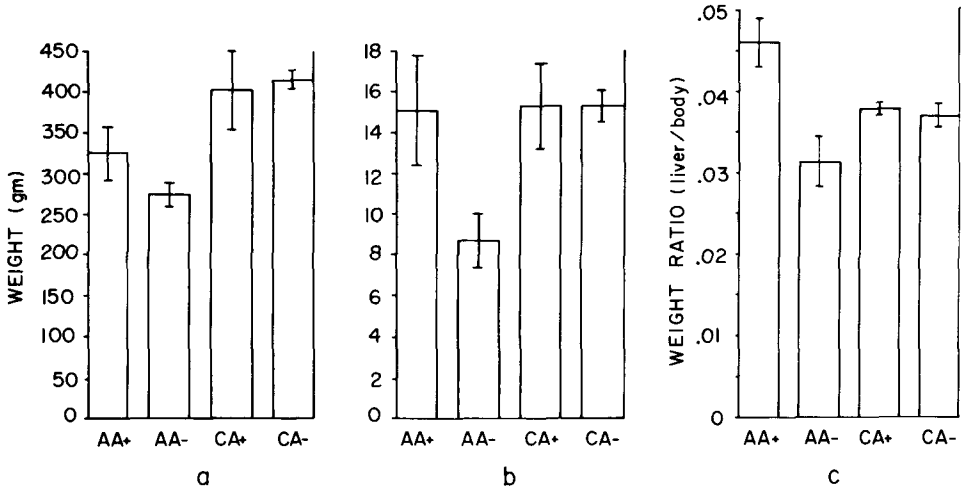

FIG. 3. Liver and body weights. Weights of (a) rats and (b) livers from rats as well as (c) the ratios of liver weights to rat body weights are shown from values taken at the end of the experiment. Rats were fed control diets with (CA+) and without (CA-) arachidonate as well as alcohol containing diets with (AA+) and without (AA-) arachidonate. Error bars indicate the standard deviation.

and hence more BHT than those in the AA- group. The increased size of livers in these instances could not have resulted from the increased consumption of BHT since a dose of this drug several orders of magnitude greater than that used in our study is needed to significantly increase liver weight (18,19).

Liver weights were normalized by expressing them as wt/g body wt (Fig. 3c). The ratios of wt of liver/body wt for rats in the AA- group were smaller than those of the controls or AA+ group (Fig. 3c). The liver-to-body wt ratio of rats from the AA- group (3.1%) was similar to that reported by others for rats which were fasted for 3 days (3.2 ± 0.1%) (20). The smaller wt ratio from rats in the AA- group may be related to their reduced diet consumption (Fig. 1).

Analysis of Lipid Content

We quantitated the levels of hepatic triglycerides and phospholipids by analyzing their fatty acid methyl esters by GLC and using methyl pentadecanoate as an internal standard (Table III). The true content of the triglycerides was not calcd: it is given as the sum of the wt of the corresponding fatty acid methyl esters, since it approximates the wt of triglycerides. For convenience, the total phospholipid content also is expressed as the sum of the wt of the constitutent fatty acid methyl esters.

When rats were fed the control diet, their livers contained only a small amount of triglyceride (7.01 mg/g wet wt; see Table III). When the control diet contained 20:4, the hepatic triglyceride content was not appreciably altered (9.9 mg/g). However, when rats were fed the alcohol diet, the amount of triglyceride in the liver increased ca. 10-fold (68.4 mg/g) and when the alcohol diet containing 20:4 was fed, a 50% decrease in the liver triglyceride content was observed.

The phospholipid content of livers from rats fed the alcohol diet without arachidonic acid (AA-) was significantly greater (Table III) than that of rats fed the control diet (CA-) as observed by others (21-24). Feeding arachi-

TABLE III

Triglyceride and Phospholipid Content of Livers from Rats Fed Various Diets

Diet[a]	Triglyceride	Phospholipid
CA-	7.01 ± 0.01	16.6 ± 1.2[c]
CA+	9.86 ± 1.47	18.9 ± 0.4
AA-	68.4 ± 6.37[b]	23.1 ± 1.3[c]
AA+	27.56 ± 4.34[b]	19.4 ± 2.4

[a]See Table I for diet abbreviations. Values sharing a common superscript are significantly different: [b]$p < 0.01$ and [c]$p < 0.05$ using the 2-tailed t-test. The content of triglycerides and phospholipids are given as mg fatty acid methyl esters/g wet wt of livers. The triglyceride and phospholipid fractions were separated from the total lipids of livers by TLC (14). These were quantitated by the analysis of their fatty acid methyl esters by GLC using methyl pentadecanoate as an internal standard. The mean ± SEM of values from separate analysis of liver lipids from 3 rats in each diet group are given.

donic acid apparently increases the phospholipid content of livers from rats fed the control diet (CA+) and reduces the phospholipid content of livers from rats fed the alcohol (AA+) diet.

Fatty Acid Composition of Liver Lipids

The fatty acid composition of total liver lipids was altered after feeding alcohol, 20:4 or both (Table IV). In total liver lipids from rats fed the alcohol diet (AA-), the relative levels of 16:0, 16:1 and 20:4 were lower and the levels of 18:1 and 18:2 were higher than in controls. The ratio of percentages of 16:0 to 16:1 was 9.7 in the liver lipids of control rats; it was 15.9 in alcohol-fed animals (Table IV). This finding indicates alcohol feeding might inhibit the hepatic desaturase activity. Such an inhibition of the desaturase would also be expected to increase the ratio of 18:0 to 18:1. However, this ratio decreased from 0.4 in controls (CA-) to 0.1 in the alcohol-fed group (AA-). The discrepancy in these findings may have been caused by the accumulation of dietary fat rich in 18:1 (Table II) in livers of rats fed the ethanol diet (AA-).

As observed previously in various tissues (6-12), the ratio of the relative amounts of 18:2-to-20:4 was significantly larger in livers of alcohol-fed rats compared to controls (Table IV). The ratio of 18:2-to-20:4 was 0.7 for controls (CA-) and 3.8 for the alcohol-fed animals (AA-). As expected, the 18:2/20'4 ratio decreased to 0.6 upon feeding arachidonate in rats fed the control diet (CA+) and to 1.4 in those fed ethanol (AA+). Furthermore, feeding arachidonic acid increased the levels of 20:4 and 22:4 (Table IV).

The hepatic triglycerides from rats fed alcohol had a fatty acid distribution different than that of controls (Table V). In this lipid fraction, the relative level of 16:0-to-16:1 was larger in ethanol-fed animals (13.2) than in controls (7.2). The proportion of linoleate was ca. twice that of the controls. The levels of 20:4 and 22:4 in liver triglycerides were small when the diets did not contain arachidonic acid. However, when arachidonic acid was included in the diet, as much as 10% of the fatty acids from liver triglycerides were 20:4 and as much as 5% were 22:4. The relative level of 18:2/20:4 decreased in this fraction ca. 10-fold from dietary 20:4.

The fatty acid composition of hepatic phospholipids was different than that of total lipids or triglycerides from the same organ (Table VI). These phospholipids contained relatively more 18:0 than 18:1. Regardless of

TABLE IV

Fatty Acid Composition of Total Lipids from Livers of Rats Fed Various Diets

Fatty acid	Diets[a]			
	CA-	CA+	AA-	AA+
14:0	T	T	T	T
16:0	21.6 ± 0.9b	19.4 ± 1.2	11.6 ± 0.5	14.3 ± 1.3
16:1	2.5 ± 0.6	1.8 ± 0.3	0.7 ± 0.1	1.6 ± 0.1
18:0	13.0 ± 1.0	9.7 ± 1.1	6.4 ± 0.2	6.8 ± 2.0
18:1	31.4 ± 1.0	28.7 ± 0.9	52.0 ± 0.2	40.3 ± 1.3
18:2	11.6 ± 1.2	13.2 ± 1.0	22.6 ± 0.7	17.2 ± 0.9
20:4	16.9 ± 1.2	21.6 ± 2.2	6.0 ± 0.5	12.6 ± 0.5
22:4	0.8 ± 0.2	3.4 ± 0.2	T	4.7 ± 1.0
22:6	1.8 ± 0.2	1.6 ± 0.3	T	1.4 ± 0.5
16:0 / 16:1	9.7 ± 2.3	11.2 ± 1.5	15.9 ± 1.2	9.1 ± 0.9
18:0 / 18:1	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.01	0.2 ± 0.1
18:2 / 20:4	0.7 ± 0.1	0.6 ± 0.1	3.8 ± 0.3[c]	1.4 ± 0.1[c]

[a]See Table I for diet abbreviations.

[b]Values are given as percentage of total fatty acids. The total lipids were extracted from rat livers (13). Their fatty acid methyl esters were prepared and analyzed by GLC. Percentage of total fatty acids are given as the mean ± SEM of values from separate analyses with 3 rats in each diet group. Values of 0.5% or less are give as T. The values for 22:4 also include 24:0 and 24:1.

[c]Values sharing a common superscript are significantly different with $p < 0.01$.

TABLE V

Fatty Acid Composition of Triglycerides from Livers of Rats Fed Various Diets

Fatty acid	Diets[a]			
	CA-	CA+	AA-	AA+
14:0	1.1 ± 0.2[b]	0.9 ± 0.2	T	T
16:0	27.2 ± 2.3	19.8 ± 0.8	11.8 ± 0.6	13.0 ± 0.3
16:1	4.1 ± 1.0	2.4 ± 0.3	0.9 ± 0.1	1.6 ± 0.02
18:0	3.9 ± 1.0	2.6 ± 1.0	2.6 ± 0.2	1.4 ± 0.03
18:1	51.2 ± 2.1	40.4 ± 0.4	59.1 ± 0.9	44.2 ± 1.3
18:2	11.1 ± 1.8	18.5 ± 1.0	22.2 ± 1.1	20.7 ± 0.7
20:4	1.2 ± 0.6	10.3 ± 0.8	1.6 ± 0.6	10.0 ± 1.0
22:4	T	4.5 ± 0.6	1.2 ± 0.3	6.3 ± 0.6
22:5	T	T	T	2.0 ± 0.4
16:0 / 16:1	7.2 ± 1.4[c]	8.5 ± 0.8	13.2 ± 0.8[c]	8.0 ± 0.1
18:0 / 18:1	0.076 ± 0.020	0.067 ± 0.027	0.044 ± 0.003	0.032 ± 0.001
18:2 / 20:4	28.8 ± 3.1	1.8 ± 0.1	17.5 ± 5.4	2.1 ± 0.2

[a]See Table I for diet abbreviations.

[b]Values are given as percentage of total fatty acids. Triglycerides were isolated from the total lipids of livers by TLC (14). Their fatty acid methyl esters were prepared and analyzed by GLC. Percentage of fatty acids given are mean ± SEM of values from separate analyses with 3 rats in each diet group. Values of 0.5% or less are given as T.

[c]Values sharing a common superscript are significantly different with $p < .05$.

TABLE VI

Fatty Acid Composition of Phospholipids from Livers of Rats Fed Various Diets

Fatty acid	Diets[a]			
	CA-	CA+	AA-	AA+
14:0	0.7 ± 0.4[b]	T	T	T
16:0	19.5 ± 0.2	18.8 ± 0.9	14.0 ± 0.9	15.7 ± 0.1
16:1	1.2 ± 0.3	0.6 ± 0.1	T	0.7 ± 0.2
18:0	22.6 ± 0.8	21.8 ± 0.2	27.9 ± 1.3	27.1 ± 0.3
18:1	16.0 ± 0.3	12.2 ± 1.0	14.7 ± 2.0	16.1 ± 1.3
18:2	10.1 ± 1.2	6.2 ± 0.4	10.5 ± 2.0	5.5 ± 1.3
20:4	24.9 ± 0.2	33.7 ± 0.5	27.4 ± 2.4	28.2 ± 1.1
22:4	1.1 ± 0.1	1.9 ± 0.1	1.2 ± 0.2	1.3 ± 0.2
22:5	1.4 ± 0.3	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.2
22:6	2.5 ± 0.2	3.9 ± 0.3	2.6 ± 0.8	4.2 ± 0.3
16:0 / 16:1	17.4 ± 4.1	34.9 ± 3.9	42.6 ± 14.0	26.7 ± 8.4
18:0 / 18:1	1.4 ± 0.1	1.8 ± 0.1	2.0 ± 0.3	1.7 ± 0.1
18:2 / 20:4	0.40 ± 0.05[c]	0.16 ± 0.03[c]	0.39 ± 0.08[d]	0.19 ± 0.05[d]

[a]See Table I for diet abbreviations.

[b]Values are given as percentage of total fatty acids. Phospholipids were separated from the total lipids of livers by TLC (14). Their fatty acid methyl esters were prepared and analyzed by GLC. Percentage of total fatty acids given are mean ± SEM of values from separate analyses with 3 rats in each diet group. Values 0.5% or less are given as T. The value for 22:4 also includes the 24:0 and 24:1.

[c]Values sharing the common superscript[c] are significantly different with $p < 0.05$.

[d] Superscript[d] indicates not significantly different ($p > 0.05$).

the dietary conditions, phospholipids contained detectable amounts of 22:4, 22:5 and a significant amount of 22:6. In animals that were fed arachidonate, the levels of 18:2 in phospholipids decreased, thereby decreasing the relative level of 18:2/20:4. The level of 20:4 in phospholipids was not significantly altered by the inclusion of arachidonic acid in the diet.

DISCUSSION

Earlier studies with dietary supplements of protein or lipotropic factors to reduce fatty liver were not successful (4). However, more recent investigations by Stanko et al. (25) reported fatty liver could be prevented by supplementing the alcohol diet with pyruvate, dihydroxyacetone and riboflavin. The addition of adenosine to the diet also has been shown to reduce hepatic triglycerides in rats fed ethanol for 8 hr (26). In this study, we fed rats an alcohol diet with (AA+) or without (AA-) arachidonic acid for 28 days and quantitated the liver triglyceride content by the analysis of fatty acids using GLC. Hepatic triglycerides were reduced by more than 50% when 20:4 was included in the alcohol diet (Table III). The amount of triglyceride/g of liver in alcohol-fed rats (68 mg/g AA-) observed in this study is similar to that reported by others (65 mg/g and 56 mg/g) in which the same diet was fed to rats but different assay techniques were used (25,27). In rats which were fed the control diet ad libitum, we found the triglyceride content of livers was very low (7.01 mg/g, Table III). DeCarli and Lieber have also reported similar low values (10.8 mg/g) in rats even when they were pair-fed a control diet identical to that used in our study (27). However, Stanko et al. (25) observed a significantly higher content of triglycerides (22 mg/g) in the livers of rats that were pair-fed this control diet. Since the content of triglycerides in animals fed alcohol, dihydroxyacetone, pyruvate and riboflavin was ca. 22-30 mg/g, it was concluded by Stanko et al. that alcohol-induced fatty liver was prevented by feeding these metabolites (25). If these triglyceride levels are compared to those from livers of rats fed a control diet either ad libitum (Table III) or pair-fed (2), one could also conclude that feeding pyruvate, dihydroxyacetone and riboflavin only reduced fatty liver by ca. 50%. This is the same reduction in liver triglycerides we observed after supplementing the alcohol diet with 20:4.

It has been generally accepted that ethanol ingestion increases not only the content of triglycerides, but also that of phospholipids and cholesteryl esters in liver (3). We analyzed results from experiments by Stanko et al. in which the concentration of the total esterified fatty acids and triglycerides in liver lipids from alcohol-fed rats were reported (25). From their results, we conclude that hepatic triglycerides accounted for nearly all of the total esterified fatty acids. Thus, the liver phospholipid content apparently decreased from feeding ethanol (25). In our studies, the hepatic phospholipid content of rats fed alcohol (AA-) was greater than that of rats fed the control diet (Table III) which has been reported by other investigators (21-24). Although we did not find statistically significant differences, feeding arachidonic acid apparently increases hepatic phospholipids in control rats and reduces them in ethanol-fed rats. Further experiments would indicate whether this trend is significant. In this study, we did not determine the content of cholesterol and cholesteryl esters from livers of rats fed the various diets. The levels of cholesterol (2 mg/g) and cholesteryl esters (3 mg/g) have been shown to be very small compared to the levels of triglycerides and phospholipids in the livers of rats fed the alcohol diet (27).

Feeding alcohol or 20:4 alters the fatty acid composition of hepatic lipids. The ratio of the relative amounts of 16:0-to-16:1 increased with ethanol feeding (Table IV). Whether this results from a decreased level of desaturase enzyme in the livers of rats fed alcohol remains to be determined.

We have demonstrated that feeding ethanol causes an increased ratio of 18:2/20:4 in liver total lipids. Such an increase in the ratio of 18:2/20:4 by ethanol feeding has been reported for various tissues by several investigators (12). It has been suggested (6,7) that the increase in this ratio is caused by a decreased synthesis of 20:4 from 18:2 by the inhibition of the elongation and desaturation system. However, the increased ratio observed in this study may have resulted from the hepatic accumulation of triglycerides (Table III) which contains large amounts of 18:2 but only trace levels of 20:4 (Table II). The relative levels of 18:2/20:4 in the liver phospholipids of control and alcohol-fed animals were similar (Table VI). Even though the level of linoleate was 2-fold greater in liver triglycerides of alcohol-fed rats than of controls, the 18:2/20:4 ratio may not be meaningful since triglycerides contained only small amounts of 20:4.

The fatty acid composition of hepatic triglycerides from alcohol-fed rats is similar to that of dietary fat (Tables II and V). This observation supports conclusions of others (21) that an increased liver triglyceride content results from an accumulation of dietary fat.

Although triglycerides contain only low levels of 20:4 and 22:4 in animals fed the control diet, the diet supplemented with arachidonic acid increases the levels of these polyunsaturated fatty acids several-fold (Table V). Since dietary fat is practically free of 22:4, this acid could have been produced from 20:4 and used in the de novo synthesis of triglycerides or incorporated by transacylation reactions with endogenous triglycerides.

During the initial 10 days of this experiment, rats fed control diets (CA- and CA+) consumed considerably more diet than those fed ethanol (AA- and AA+) (Fig. 1). Subsequently, in the alcohol-fed groups, dietary 20:4 (AA+) caused the animals to consume more diet and to gain more wt than those not fed arachidonic acid (AA-). In the same period, dietary 20:4 did not significantly alter either diet consumption or wt of control animals (Figs. 1 and 2). The mechanisms responsible for the initial lag in the food consumption of the rats in the AA+ group and the subsequent stimulation of their appetite by 20:4 are unknown. At the end of the experiment, the diet consumption and rate of wt-gain in animals fed ethanol and arachidonic acid were approaching corresponding values for controls.

Rats fed the alcohol diet (AA-) had livers which weighed only about half as much as those on control diets (Fig. 3a). In rats fed the alcohol diet containing 20:4 (AA+), liver weights were comparable to those of controls. Such an increase in liver size induced by this inclusion of 20:4 in the ethanol diet may be related to the amount of diet consumed, and could result from an increased number of hepatic cells or cellular constituents.

Dietary 20:4 is known to inhibit hepatic lipogenesis (5). It also can stimulate the hepatic syntheis of phospholipids since it is a naturally occurring fatty acid in this lipid class. Since phospholipids are necessary components of lipoproteins, hepatic synthesis of these lipids must have a role in the mobilization of fat. Therefore, we expected that feeding rats arachidonic acid with ethanol would decrease hepatic lipogenesis and increase fat mobilization from liver—both processes contributing toward a reduction of fatty liver. We have observed a reduction in the liver content of triglycerides in alcohol-fed rats induced by dietary 20:4. Our results also indicate the origin of the triglycerides in fatty liver is dietary fat. Therefore, decreased hepatic lipogenesis may not play a major role in the reduction of fatty liver. Thus, mobilization of fat from the liver may have increased when rats were fed the alcohol diet containing 20:4. Further studies on the analysis of plasma triglyceride contents and lipogenic capacities of livers are necessary to determine the mechanisms involved in reducing the level of hepatic triglycerides in rats fed alcohol and arachidonic acid.

In this study, we have shown that dietary 20:4 causes an increase in diet consumption as well as body and liver wt gain in ethanol-fed rats. These observations were made after feeding the diets ad libitum whereas with other methods, such as pair feeding, the same results would not have been possible. Whether fatty liver is reduced in animals fed the alcohol diet supplemented with arachidonic acid by means other than ad libitum remains to be determined.

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Effects of an Essential Fatty Acid Deficiency on Serum Lipoproteins in the Rat

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ABSTRACT

Studies are reported on the effect of an essential fatty acid (EFA) deficiency in male Sprague-Dawley rats and its exacerbation by inclusion of *trans* fatty acids in the diet on the level and composition of serum lipoproteins. Weanling male Sprague-Dawley rats were fed diets containing all essential nutritients and a 5% fat supplement of safflower oil (SAFF) or hydrogenated coconut oil (HCO) in 2 experiments, one for 31 wk and the other for 17 wk. For the final 3 wk of each experiment, animals were switched from each group to a 5% supplement of a concentrate of ethyl linolelaidate (TRANS). In addition, a group of animals fed the HCO diet in the first experiment were also switched to the SAFF Diet. With the development of an EFA deficiency in the HCO group, there was a decrease in the high density lipoprotein (HDL) and an increase in the very low density plus the low density (VL-LDL) lipoprotein fractions separated by heparin-manganese precipitation. Switching animals of the HCO group to the TRANS supplement exaggerated this effect and produced a very low ratio of HDL-to-VL-LDL. Analysis of the serum lipoproteins by polyacrylamide disc gel electrophoresis showed that an EFA deficiency produced a marked alternation of the HDL fraction. Changes also appeared to be produced in the VL-LDL fraction by an EFA deficiency and particularly upon switching EFA-deficient animals to the TRANS supplemented diet. Switching animals of the SAFF group to the TRANS supplement brough about an immediate reduction in HDL with a corresponding decrease in serum arachidonic acid. The data suggested a general relationship between arachidonic acid and the level and composition of HDL on the one hand, and 18:1 and VL-LDL on the other. Accordingly, the ratio of HDL-to-VL-LDL appears to provide a sensitive biochemical index of the EFA status of the rat.

INTRODUCTION

Effects of an essential fatty acid (EFA) deficiency in the rat has been studied extensively and changes in fatty acid composition of the tissues have been well characterized (1). A deficiency of EFA also affects the activities of enzymes involved in lipid metabolism and transport, particularly lipoprotein lipase (2,3) and lecithin cholesterol acyl transferase (3,4), as well as the hepatic synthesis of fatty acids (5,6). As a consequence of these effects, fat accumulates in the liver and the level of serum lipid is decreased in an EFA deficiency (7-10). Previous studies (11,12) in this laboratory indicated that accumulation of fat in the liver of EFA-deficient rats also was caused by impaired secretion of lipid into the blood, indicating a defect in lipoprotein synthesis. Studies are reported here on effects of an EFA deficiency and its exacerbation by dietary *trans* fatty acids on the level and composition of serum lipoproteins.

EXPERIMENTAL

Animals

Weanling male rats of the Sprague-Dawley strain, 50-60 g (ARS Sprague-Dawley Corp., Madison, WI), were housed in individual cages and fed ad libitum a fat-free diet containing all essential nutrients (Table I) and 5% fat of different compositions (Table II) as described in the following experiments.

Two experiments were performed. In the first experiment, animals were fed diets containing a supplement of 5% hydrogenated coconut oil (HCO group) or 5% safflower oil (SAFF group) (Table II). After ca. 28 wk, 6

TABLE I

Diet Composition, Percent by Weight

Casein vitamin test	22.5	Choline mix[a]	1.0
Wesson salt mixture[b]	4.0	Cellulose alphacel	10.5
Vitamin mix[c]	1.0	Fat	5.0
Sucrose	56.0		

[a]Choline mix consists of 22% choline dihydrogen citrate in vitamin test casein.

[b]Wesson salt mixture does not contain zinc or manganese, hence these elements are added to the mix as follows: $ZnCl_2$ (0.60 g) and $MnSO_4 \cdot H_2O$, 0.90 g/200 g of salt mixture.

[c]Fat and vitamins A, D and E are mixed into the diet daily and stored at 0 C overnight. Vitamin D_2, 5.0 mg; retinol acetate, 6.9 mg; α-tocopherol acetate, 300 mg/kg of diet. Vitamin mix (g): thiamine HCL, 2.5; riboflavin, 2.5; nicotinic acid, 9.0; calcium pantothenate, 9.0; pyridoxine HCl, 2.0; cyanodobalamin (B_{12}), 4.0; *p*-aminobenzoic acid, 7.5; folic acid, 0.1; biotin, 0 0.02; *meso*-inositol, 20.0; menadione (vitamin K), 0.5; vitamin test casein, 943.0.

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TABLE II

Fatty Acid Composition of Dietary Fats[a]

Group I, 5% SAFF[b]			Group II, 5% HCO			Group III, 5% HCO-TRANS[c]		
Fatty acid[d]	Fat (% wt)	% Dietary calories	Fatty acid	Fat (% wt)	% Dietary calories	Fatty acid	Fat (% wt)	% Dietary calories
16:0	9.6	1.2	10:0	3.5	0.4	16:0	18.1	2.3
18:0	3.6	0.5	12:0	46.9	5.9	18:0	7.3	0.9
18:1	13.4	1.6	14:0	20.2	2.5	*t*-18:1	24.3	3.0
18:2	73.4	9.2	16:0	11.0	1.4	*t,t*-18:2	50.3	6.3
			18:0	16.0	1.9			
			18:1	2.3	0.3			

[a]Groups I, II and III are the same for both experiments I and II.

[b]SAFF = safflower oil, HCO = hydrogenated coconut oil, TRANS = ethyl linolelaidate concentrate.

[c]Group III, animals shifted from 5% HCO to 5% TRANS supplement for final 3 wk; group IV, experiment I, SAFF-TRANS had same final composition as group III; group V, experiment I, HCO-SAFF and same final composition as group I.

[d]Shorthand designation for fatty acids. Number before colon = chain length, number after colon = number of double bonds.

animals of each group were switched to a supplement of a 5% concentrate of ethyl linolelaidate (TRANS) (Table II). At the same time, 6 animals of the HCO group were switched to the SAFF supplement giving a total of 5 groups in this experiment: group I, SAFF; group II, HCO; group III, HCO-TRANS; group IV, SAFF-TRANS; group V, HCO-SAFF. At the end of the 31st wk, blood was withdrawn from the retro-ocular plexes of the animals of each group for analysis of the serum and lipoprotein lipids.

In the second experiment, one group of rats was fed the HCO supplement and another the SAFF supplement from weaning for 14 wk. Then 6 animals of the HCO group were switched to the TRANS supplement. At the end of 17 wk, the serum and lipoprotein lipids were analyzed on blood withdrawn from the retro-ocular plexes of the animals of the 3 groups: group I, SAFF; group II, HCO; group III, HCO-TRANS.

Lipid Analysis

Thin layer chromatography (TLC) was used for the analysis and fractionation of the lipid classes as previously described (4). The neutral lipids were separated into cholesteryl esters (CE), triglycerides (TG), free fatty acids and cholesterol using a double development technique in which the plate was developed first with a solvent of petroleum ether/ethyl ether (90:10, v/v) followed by petroleum ether/ethyl ether/acetic acid (80:20:0.2, v/v) to a second front 5 cm below the first. The polar lipids (PL) separated as a fraction at the origin of the plate. This fraction consists almost entirely of phospholipids in rat serum, and the PL designation in this text refers generally to these compounds.

Fatty acid composition was determined by gas liquid chromatography (GLC) of methyl esters prepared by interesterification with methanol using HCl as the catalyst (13). The GLC was carried out with a Hewlett-Packard Model 5840A instrument equipped with dual flame detectors and a column of 10% Silar 10C on Gas Chrom Q, mesh 100-200. Helium was used as the carrier gas at a flow rate of 8.5 ml/min and the temperature was programmed from 200 to 250 C at 2 C/min.

Lipoprotein Fractionation and Analysis

Polyacrylamide disc gel electrophoresis was performed as described by Narayan (14) using a 3.75% gel. In this procedure, serum (0.2 ml) was prestained with Sudan Black (0.1 ml), 1% solution in ethylene glycol. After a 1-hr incubation period at room temperature in the dark, a sucrose solution (0.3 ml) was added and 30 μl, which corresponded to 10 μl of serum, was applied to the gel. The bands were designated according to Narayan (14) in that virtually identical results were obtained with normal rat serum.

Fractionation of the serum lipoproteins was carried out by heparin-manganese precipitation essentially as described by Burstein et al. (15) as follows: 60 μl of heparin solution (5000 USP K units/ml, Sigma Chemical Co., St. Louis, MO) and serum (1.5 ml) were mixed together in a 1.5-ml centrifuge tube. Then a 1.0 M manganese chloride solution (90 μl) was added to the tube. After the solution was thoroughly mixed, it was allowed to stand for 30 min at 4 C to precipitate the very low density plus the

low density lipoprotein fraction (VL-LDL) and centrifuged at 1600 x g for 20 min. An aliquot of the supernatant was taken for analysis of high density lipoproteins (HDL). The precipitate which contained the VLDL plus LDL was separated, washed twice with a solution of 0.1% heparin and 0.05 M manganese chloride to remove the occluded mother liquor. The lipids were extracted from each fraction with chloroform/methanol (2:1, v/v) and purified by redissolving them in low boiling petroleum ether after evaporation of the chloroform and methanol under reduced pressure at room temperature. The separation of the 2 lipoprotein fractions was monitored by analysis of the lipid by TLC and polyacrylamide disc gel electrophoresis as illustrated with the serum of a normal animal in Figure 1. These analyses indicated that there was no overlapping of the 2 fractions inasmuch as no free fatty acid normally associated with albumin was detected in the VL-LDL fraction and no TG was detected in the HDL fraction (Fig. 1a). Electrophoresis of the redissolved VL-LDL fraction showed that it was devoid of HDL as illustrated in Figure 1b.

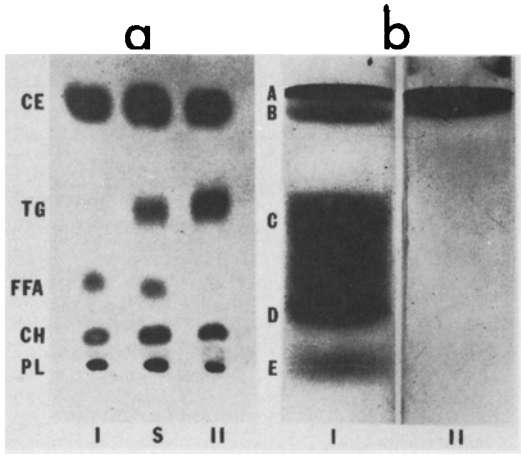

FIG. 1. (a) Thin layer chromatographic analyses of: I, HDL lipid of normal rat serum. S, standard mixture; CE, cholesteryl ester; TG, triglyceride; FFA, free fatty acid; CH, cholesterol; PL, phospholipid (dipalmitoyl phosphatidylcholine). II, redissolved very low density lipoprotein plus low density lipoprotein (VL-LDL) fraction isolated by heparin-manganese precipitation from normal rat serum. (b) Polyacrylamide disc gel electrophoresis, 3.75% gel. I, Prestained serum of normal rats with Sudan Black B. A, chylomicron plus VLDL; B, LDL: C, HDL_1 + HDL_3; D, HDL_2; E, albumin. II, redissolved very low density plus low density lipoprotein (VL-LDL) fraction isolated by heparin-manganese precipitation prestained with Sudan Black B. A, chylomicron plus VLDL; B, LDL.

RESULTS

Effects of the dietary supplements on fatty acid composition and levels of the HDL and VL-LDL fractions in experiment 1 are shown in Table III and Figure 2, respectively. The diet of group I contained 9.2% of the calories as linoleic acid, which is well in excess of the EFA requirement. Accordingly, the fatty acid composition and lipoprotein levels were assumed to have normal distribution patterns. Arachidonic acid was concentrated more in HDL than in VL-LDL; linoleic acid was higher in VL-LDL than HDL. The 18:1 was also higher in VL-LDL than in HDL, but 18:0 was higher in HDL. Only trace amounts of 16:1 were present in either fraction, but 16:0 was higher in the VL-LDL fraction. HDL was much higher than the VL-LDL fraction in this group (I) and the ratio of these fractions was 2.44 (Fig. 2).

Group II, which received the HCO supplemented diet, exhibited the typical fatty acid compositional pattern of an EFA deficiency in the serum (as also shown in Table III), that is, low 18:2 and 20:4, elevation of 16:1 and 18:1 and a high concentration of 20:3ω9. The 20:3ω9 was concentrated more in the HDL fraction similarly to arachidonic acid in group I; 18:0 and 18:1 also were distributed between HDL and VL-LDL similarly to group I. However, 16:0, which was greatly decreased, and 16:1, which was increased, were distributed

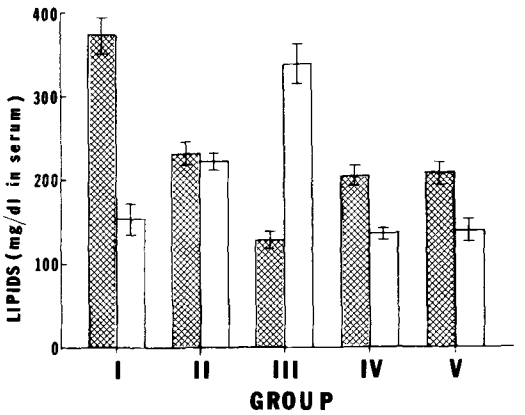

FIG. 2. Effect of dietary regimens in experiment I on serum HDL and very low density plus low density (VL-LDL) lipid fractions isolated by heparin-manganese precipitation. Hatched bars, HDL; open bars, VL-LDL fraction, I, SAFF group; II, HCO group; III, HCO-TRANS group. IV, SAFF-TRANS group; V, HCO-SAFF group. HDL, I vs II, $P < 0.001$; I vs III, $P < 0.001$; I vs IV, $P < 0.001$ and II vs V, ns. VL-LDL, I vs II, $P < 0.005$; I vs III, $P < 0.001$; I vs IV, ns; II vs V, $P < 0.001$.

TABLE III

Experiment I: Fatty Acid Composition of Serum and Lipoprotein Lipid (% by wt)

	16:0	16:1	18:0	18:1	*t,t*-18:2	*c,c*-18:2	20:3	20:4
Group I: SAFF diet[a]								
Serum	34.2 ± 5.1[b]	0.9 ± 0.8	14.9 ± 4.6	6.3 ± 2.0		15.6 ± 1.0		21.0 ± 1.1
HDL[c]	18.8 ± 2.2	1.5 ± 0.1	13.6 ± 2.1	6.5 ± 0.4		16.3 ± 0.4		40.7 ± 4.5
VL-LDL[d]	31.6 ± 2.2	1.9 ± 1.2	7.3 ± 0.4	14.6 ± 1.5		30.1 ± 1.7		9.8 ± 1.0
Group II: HCO diet								
Serum	19.0 ± 0.5	10.6 ± 0.3	8.5 ± 0.3	43.7 ± 1.8		tr	14.2 ± 1.3	0.4 ± 0.2
HDL	19.2 ± 1.6	9.8 ± 1.5	11.4 ± 0.3	30.6 ± 0.4		tr	24.6 ± 1.9	2.4 ± 1.9
VL-LDL	19.3 ± 1.7	10.7 ± 0.9	7.6 ± 0.6	48.2 ± 2.2			10.3 ± 0.2	0.3 ± 0.1
Group III: HCO-TRANS diet								
Serum	20.5 ± 2.1	8.5 ± 4.8	6.1 ± 0.8	54.4 ± 0.7	2.9 ± 0.6	1.4 ± 0.1	2.3 ± 0.1	0.3 ± 0.2
HDL	25.4 ± 3.2	9.2 ± 1.7	8.5 ± 1.8	50.0 ± 3.0	tr	1.6 ± 0.3	5.7 ± 1.9	0.3 ± 0.1
VL-LDL	19.5 ± 0.5	11.8 ± 2.0	6.1 ± 0.9	56.1 ± 1.3	tr	1.5 ± 0.1	2.1 ± 0.3	0.2 ± 0.1
Group IV: SAFF-TRANS diet								
Serum	29.7 ± 3.8	4.0 ± 0.5	8.5 ± 0.6	19.6 ± 2.7	2.6 ± 0.5	18.6 ± 0.5		11.9 ± 2.6
HDL	24.6 ± 1.4	3.2 ± 0.8	12.5 ± 3.1	12.3 ± 1.6	1.9 ± 0.2	14.7 ± 1.0		23.9 ± 1.2
VL-LDL	26.3 ± 0.1	4.1 ± 0.4	5.1 ± 1.1	25.4 ± 0.8	3.4 ± 0.6	24.6 ± 2.1		6.3 ± 0.5
Group V: HCO-SAFF diet								
Serum	31.0 ± 5.0	4.8 ± 0.5	12.2 ± 1.2	21.8 ± 2.7		13.8 ± 0.8		14.0 ± 4.6
HDL	29.0 ± 5.9	4.3 ± 0.6	14.6 ± 1.3	14.6 ± 0.7		11.6 ± 0.6		22.4 ± 4.5
VL-LDL	24.4 ± 0.3	4.6 ± 0.6	7.9 ± 0.9	27.0 ± 1.6		16.5 ± 0.8		12.8 ± 1.7

[a]See text for details of dietary regimens of each group.

[b]M ± SE.

[c]HDL = high density lipoprotein fraction.

[d]VL-LDL = very low density lipoprotein plus low density lipoprotein fractions.

equally between HDL and VL-LDL in this group.

The effect of the HCO dietary fat was to lower the level of HDL and increase the level of VL-LDL thereby reducing the ratio of these fractions to ca. 1.0 as shown in Figure 2.

Switching animals of group II to the TRANS supplement (group III) for the relatively short period of 3 wk exaggerated the effect of the HCO diet on the composition of the serum lipoproteins as shown in Figure 2. HDL was decreased further and the VL-LDL fraction was greatly increased compared to the SAFF group, producing a further reduction of the ratio of HDL-to-VL-LDL to ca. 0.38. Examination of the fatty acid composition of the sera and lipoprotein fractions of the animals of this group showed that these changes were accompanied by a large increase in 18:1 with a corresponding large decrease in 20:3ω9 (Table III). The TRANS supplement also appeared to influence the normal pattern of the distribution of 18:1, 18:0 and 16:0 between HDL and VL-LDL inasmuch as the differences in the distribution of these acids were relatively minor. Linolelaidic acid (*t,t*-18:2) was detected in the serum lipids of this group but it was a relatively minor constituent. No elaidic acid was detected in the serum lipids at the level fed, ca. 3% of the dietary calories.

The animals of group IV, which were switched from the SAFF to the TRANS supplemented diet for the final 3 wk of the experiment, exhibited a decrease of ca. 50% in the level of arachidonic acid in the serum and lipoprotein lipoprotein lipids (Table III). The percentage of 18:1 was increased several-fold; however, there was little change in the level of linoleic acid. There was essentially no change in the level of VL-LDL in the animals of this group compared tothe SAFF group from which they were switched, but the HDL fraction was greatly reduced (Fig. 2). Accordingly, the ratio of HDL-to-VL-LDL was reduced from 2.44 in the SAFF group to 1.5.

Switching the animals from the HCO diet (group II) to the SAFF supplemented diet reversed the fatty acid composition of the serum toward normal. The formation of 20:3ω9 was completely suppressed, arachidonic acid was increased to ca. 67% of that of the control SAFF group I, and the levels of 16:1 and 18:1 were reduced to ca. 50% of that of the parent HCO group. Simultaneously, there was a reduction of the VL-LDL fraction but there was no change in the level of HDL. The ratio of HDL-to-VL-LDL was increased accordingly to 1.5 from 1.02 in the parent

HCO group.

Effects of the dietary supplements on the fatty acid composition of the serum lipoproteins of the animals in the second experiment are reported in Table IV. In general, the composition of the fatty acids in this experiment followed the same pattern in the serum and lipoprotein fractions as corresponding groups in the first experiment. Examination of the distribution of the fatty acids between the cholesteryl esters and phospholipids also showed particular patterns. Arachidonic and linoleic acids were concentrated in the cholesteryl esters, and 16:0 and 18:0 in the phospholipids of the SAFF group. This pattern was also followed generally in the HCO group and in group III inasmuch as 16:1 and 18:1 were concentrated in the cholesteryl esters.

As in the first experiment, the ratio of HDL-to-VL-LDL was reduced by an EFA deficiency (HCO group) and further reduced upon switching the animals of this group to the TRANS supplemented diet as shown in Table IV.

The effect of the development of an EFA deficiency (group II) and its intensification by the TRANS supplemented diet (group III) on lipoprotein composition was readily demonstrated by polyacrylamide disc gel electrophoresis analysis as shown in Figure 3. These analyses show that an EFA deficiency produced a marked effect on the HDL fraction since HDL_1 and HDL_3 were virtually absent in the animals of the HCO group. Moreover, the spreading of the HDL_2 band indicated that it also had been affected. Switching the HCO group to the TRANS diet reduced the HDL fraction to the point that it was barely detectable using the same amount of serum for the analysis. However, a weak band was present in the HDL_2 region indicating that the TRANS diet had intensified the EFA deficiency produced by the HCO diet.

Although the analyses in Figure 3 were directed to an examination of the HDL fraction, they also indicated that changes occurred in the composition of the VL-LDL fractions in both the HCO and TRANS groups, particularly in the TRANS group. The band for the LDL fraction was more intense in the analysis of the serum of the HCO group (II) than either the SAFF (I) or the TRANS (III) groups. Further, the VLDL band at the juncture of the spacer and main gels which corresponds to the VLDL species generally isolated by ultracentrifugation was relatively weak. Thus, although the intense band at the top of the spacer gel contained excess dye and probably some chylomicrons, it also contained a large amount of other lipoproteins of very low densities, especially in the animals of the TRANS group III, in accordance with the amount of VL-LDL fraction reported in Table IV.

DISCUSSION

This study shows that an EFA deficiency produces a marked effect on the level and composition of the circulating lipoproteins in the rat. In general, the HDL was decreased and the VL-LDL fraction was increased, a relationship which was intensified by switching the animals from the HCO to the TRANS diet. In addition to an effect on the concentration, there also were differences among the groups in the composition of the lipoproteins as demonstrated by the electrophoresis analyses. These analyses showed that the bands for HDL_1 and HDL_3 were greatly diminished by an EFA deficiency as also observed by Narayan (14,16). The spreading and intensity of the bands in the HDL_2 region indicated further that changes also occurred within these components, as might be expected from the large differences in fatty acid composition between the animals of the HCO and SAFF groups.

In addition to changes in HDL, there also was an effect of an EFA deficiency on the VL-LDL fraction, particularly on VLDL. A major change in this fraction appears to be a large increase in the amount of sample separated by the spacer gel. In fact, it is this fraction that accounts for the large increase in the VL-LDL fraction inasmuch as the VLDL which separated at the juncture of the spacer and main gels is relatively small. Moreover, de Pury and Collins (17) have shown that the VLDL fraction isolated by ultracentrifugation, which corresponds to this band (14), is actually decreased in an EFA deficiency. While normally the fraction at the top of the spacer gel consists mostly of chylomicrons, there should be little of these compounds in the sera in our experiments because the animals are fasted for at least 12 hr before being sacrificed. Thus, it appeared that this fraction consisted of undefined species of lipoproteins of very low densities that were produced by the EFA deficiency and also upon switching EFA-deficient animals to the TRANS supplemented diet.

A major difference between the TRANS and HCO supplemented diet that might account (at least in part) for the large difference in the amount of the VL-LDL fraction in the serum of these groups is the effect of these supplements on lipoprotein lipase activity. In previous work (3), it was shown that extrahepatic lipoprotein lipase activity was elevated in an EFA defi-

TABLE IV

Experiment II: Fatty Acid Composition of Serum Lipids (% by wt)

	g/dl	16:0	16:1	18:0	18:1	*t,t*-18:2	*c,c*-18:2	20:3ω9	20:4
Group I: SAFF diet[a]									
Serum lipid	287 ± 3[b]	19.7 ± 0.9	1.0 ± 0.02	11.8 ± 1.2	5.1 ± 0.3		24.4 ± 1.3		32.3 ± 0.7
HDL lipid[c]	183 ± 7	17.9 ± 0.7	1.0 ± 0.10	13.2 ± 0.7	4.1 ± 0.1		17.9 ± 1.0		39.6 ± 1.5
HDL CE		7.5 ± 0.4	0.7 ± 0.03		1.7 ± 0.1		14.7 ± 0.6		74.6 ± 0.5
HDL PL		27.9 ± 1.5	0.8 ± 0.05	25.6 ± 1.0	3.7 ± 0.4		11.1 ± 1.4		21.4 ± 0.9
VL-LDL lipid[d]	106 ± 5	17.9 ± 0.5	0.9 ± 0.10	8.2 ± 0.3	6.9 ± 0.4		34.8 ± 0.7		24.6 ± 0.7
VL-LDL CE		8.1 ± 0.8	1.2 ± 0.02	0.9 ± 0.1	8.1 ± 0.4		29.5 ± 1.3		51.0 ± 2.0
VL-LDL PL		24.5 ± 1.0	0.7 ± 0.02	26.2 ± 0.9	3.5 ± 0.01		11.1 ± 0.4		23.7 ± 0.6
HDL/VL-LDL	1.73								
Group II: HCO diet									
Serum lipid	234 ± 34	22.3 ± 1.2	7.1 ± 0.5	10.8 ± 1.6	32.7 ± 1.4		1.7 ± 0.3	15.2 ± 2.3	3.4 ± 0.4
HDL lipid	141 ± 26	20.6 ± 1.5	7.4 ± 0.7	12.9 ± 0.4	23.1 ± 1.2		2.0 ± 0.2	20.3 ± 1.6	4.8 ± 0.5
HDL CE		8.1 ± 1.2	13.0 ± 1.3	1.3 ± 0.4	22.2 ± 0.9		3.9 ± 0.2	32.5 ± 2.6	11.7 ± 1.2
HDL PL		23.9 ± 2.5	2.4 ± 0.5	23.6 ± 1.7	17.3 ± 1.8		1.6 ± 0.2	18.8 ± 0.9	3.1 ± 0.3
VL-LDL lipid	104 ± 26	24.0 ± 0.7	7.6 ± 0.6	7.7 ± 0.9	41.9 ± 1.4		1.3 ± 0.03	8.9 ± 0.8	1.6 ± 0.2
VL-LDL CE		15.4 ± 1.5	14.4 ± 0.4	3.0 ± 0.2	43.2 ± 2.5		2.0 ± 0.2	12.4 ± 2.9	4.7 ± 1.0
VL-LDL PL		21.4 ± 2.3	1.9 ± 0.5	25.0 ± 1.3	16.0 ± 0.9		1.5 ± 0.1	19.7 ± 1.5	3.9 ± 0.2
HDL/VL-LDL	1.36								
Group III: HCO-TRANS diet									
Serum lipid	191 ± 7.0	21.2[e] ± 1.9	10.2 ± 1.0	6.5 ± 1.0	45.8 ± 0.2	4.8 ± 0.1	3.8[f] ± 0.6	3.6 ± 0.7	1.1 ± 0.1
HDL lipid	57 ± 13	23.1 ± 0.4	10.9 ± 1.0	8.0 ± 0.1	37.5 ± 0.6	3.9 ± 0.1	4.4 ± 0.3	6.5 ± 0.8	1.9 ± 0.1
HDL CE		12.8 ± 2.1	16.4 ± 1.6	1.3 ± 0.4	43.4 ± 0.6	1.4 ± 1.1	6.3 ± 0.4	13.3 ± 0.4	5.2 ± 0.1
HDL PL		26.9 ± 2.3	5.5 ± 1.0	15.2 ± 2.3	32.5 ± 3.9	3.0 ± 0.4	5.4 ± 0.4	8.5 ± 0.3	1.8 ± 0.1
VL-LDL lipid	125 ± 1	20.7 ± 1.7	10.2 ± 1.1	6.1 ± 1.0	47.8 ± 0.6	5.0 ± 0.1	3.3 ± 0.3	2.9 ± 1.1	0.8 ± 0.2
VL-LDL CE		13.9 ± 0.1	13.8 ± 0.1	2.9 ± 0.3	54.9 ± 0.9	5.9 ± 0.1	2.8 ± 0.3	1.2 ± 0.1	0.4 ± 0.1
VL-LDL PL		23.4 ± 1.3	3.2 ± 0.1	18.3 ± 1.4	25.3 ± 0.6	3.0 ± 0.2	4.9 ± 0.1	9.8 ± 1.1	2.7 ± 0.5
HDL/VL-LDL	0.26								

[a]See text for details of dietary regimens of each group.

[b]M ± SEM of 3 samples each pooled from 2 animals.

[c]HDL = high density lipoprotein fraction.

[d]VL-LDL = very low density plus low density lipoprotein fraction.

[e]Average of 2 samples, each pooled from 2 animals.

[f]18:2 positional and geometric isomers.

ciency and that the activity of this enzyme was suppressed upon shifting animals with an EFA deficiency to a *trans* fatty acid diet. Therefore, one might expect the VLDL to be cleared from the circulation faster in the EFA-deficient animals than those fed the TRANS diet in accordance with the data shown in Figure 2 and Table IV.

The level and composition of lipoproteins are obviously related to the fatty acid composition of the serum and tissues. The nature of the changes in fatty acid composition produced by an EFA deficiency was indicated by switching animals fed the HCO and SAFF diets to the TRANS supplemented diet. This fat inhibits the conversion of oleic to 20:3ω9 in the EFA-deficient animal as well as linoleic to arachidonic acid (3,4,18,19). With the inhibition of the synthesis of 20:3ω9 as a result of the suppression of 6-desaturase activity by linolelaidate (20), there is a buildup of 18:1 as shown in this and other studies (3,4). Guo and Alexander (21) have demonstrated that 18:1 is produced by de novo synthesis from acetate derived from the catabolism of linolelaidic acid. Thus, dietary *trans* fatty acids apparently do not influence the de novo synthesis of fatty acids or the 9-desaturation of 18:0. In fact, in view of the large increase in 18:1 in the sera of the animals switched from the EFA-deficient to the TRANS diet, these reactions may be enhanced inasmuch as they are elevated in an EFA deficiency (21,22).

Switching the SAFF group to the TRANS diet for 3 wk decreased the level of arachidonic acid concomitant with a decrease in HDL; the level of VL-LDL fraction was relatively unchanged. Hence, it appeared that the effect on HDL was associated with the decrease in arachidonic acid since the level of linoleic acid remained essentially unchanged. Switching the EFA-deficient animals (group II) to the SAFF diet, which is rich in linoleic acid, started to reverse the EFA deficiency as evidenced by the changes in fatty acid composition and the increase in the ratio of HDL-to-VL-LDL. However, in this case, the change in lipoproteins involved mainly the VL-LDL fraction which was decreased to approximately normal. There was little change in the level of LDL from that in the parent HCO group in spite of the increased arachidonic acid in this group. Thus, it appeared that in these animals the composition of HDL changed with little effect in its concentration in the circulation. In agreement with these observations, it appears that the level of HDL had not increased in these animals because they were at an intermediate stage in their return to normal. Presumably,

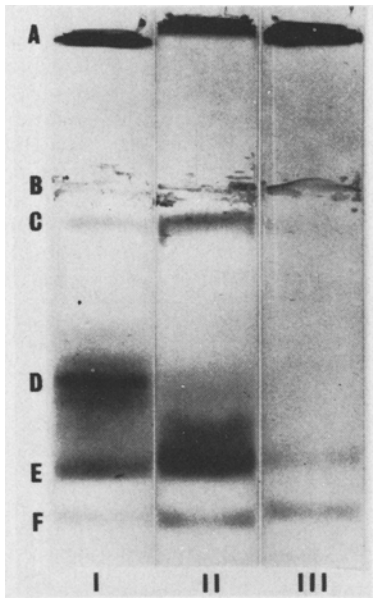

FIG. 3. Polyacrylamide disc gel electrophoresis of whole serum prestained with Sudan Black B. Experiment II: I, SAFF group; II, HCO group; III, HCO-TRANS group. A, chylomicron fraction; B, VLDL; C, LDL; D, HDL_1 plus HDL_3; E, HDL_2; F, albumin.

the level of HDL was still low because it was being rapidly used in the peripheral tissues for the replenishment of arachidonic acid. Accordingly, the ratio of HDL-to-VL-LDL was low, showing that the animals of this group had not completely recovered from an EFA deficiency.

When rats are fed linolelaidate or a concentrate devoid of *cis,cis*-linoleate as the sole source of fat in the diet, not only are the tissues depleted of arachidonic acid but the formation of 20:3ω9 is suppressed giving an unreal triene:tetraene ratio relative to the EFA status of the animals, as pointed out in early work by Privett and Blank (19). These investigations also showed that *cis*-9-*trans*-12-linoleate (octadecadienoate) undergoes interconversion to a *trans* isomer of arachidonic acid (18,19,23). Therefore, because *cis,trans*-linoleate was devoid of EFA activity (24), it was speculated particularly from data on kidney lipid that the triene:tetraene ratio might be reversed without curing an EFA deficiency (19). However, recent studies (3,4) indicate that not only does the triene:tetraene ratio of the lipid differ from tissue to tissue but in the different lipid classes. For example, virtually no 20:3 accumulates in the cholesteryl esters of the liver of EFA-deficient rats, although the level in the total lipid is high. It is evident from these observa-

tions and the recent review by Lundberg (25) of the parameters used as a biochemical index of EFA status that none is completely satisfactory.

This study shows generally that an EFA deficiency has an opposing effect on HDL and the VL-LDL fractions and that the ratio of these fractions is summarily affected. Hence, although the present work must be regarded as preliminary, it appears that the ratio of HDL-to-VL–LDL might serve as a sensitive biochemical index of EFA status.

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Composition of the Lipids in Human Milk: A Review[1]

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ABSTRACT

Recent publications on the composition of human milk are reviewed. The importance of proper sampling is discussed. Fat contents of 2.6-4.5% and cholesterol amounts of 200-650 mg/100 g fat were reported. The phytosterols in milk were increased by the consumption of these sterols. Phytosterols could contribute to the "total cholesterol" in milk if analyses are done colorimetrically. The fatty acid composition is remarkably uniform unless bizarre diets are consumed; the amounts of linoleic acid vary the most. Phospholipids contained more long chain polyunsaturated fatty acids than triacylglycerols.

INTRODUCTION

During the preparation of an earlier review on the lipids of human milk, we were astonished at the lack of reliable data on both the amount and composition of the lipids (1). Furthermore, there were very few studies in which sampling was carefully controlled and modern techniques for lipid analysis were used.

Fortunately, there has been a resurgence of interest in breast feeding, which has led to the initiation of research on the composition of human milk and the establishment of several nationally supported projects on the subject.

In this paper, we review and discuss the reports on human milk lipids that have appeared since the preparation of our review (1). Other useful, recent, general publications are an annotated bibliography (2) and books (3-5).

SAMPLING

The proper sampling of human milk has been badly neglected and improperly done as described in many papers. Samples have been taken at the beginning, middle or end of nursing, without regard for the change in total lipid content that occurs, for example, from 1.2-12.1% (6). The fatty acid composition of the total milk lipids is not altered, but the absolute amounts of the various lipid classes increase and we do not known if the relative quantities of the lipids change during the course of nursing.

A useful sampling protocol has been published (7). The entire contents from one breast were obtained by hand or with an electric pump. The electric pump is preferable because expression by hand is tedious and may not yield a total sample. The Egnell (Egnell, Inc., 412 Park Ave., Cary, IL) breast pump appears to be the best instrument for obtaining a representative sample. Although relatively expensive, the pump pulsates, can be controlled by the donor and the functional parts can be sterilized. Even though the infant may not completely empty one breast or may be transferred to the other during a nursing, a representative sample should be obtained by the complete emptying of one breast for purposes of analysis. A general questionnaire and dietary recall forms should also be used to obtain important background information.

For the analysis of lipid composition, the sample should either be analyzed immediately or frozen at -20 C and retained. Our experience has been that most samples are taken at home with the Egnell pump, placed immediately with dry ice in a Styrofoam container and then stored at either -25 or -75 C until extracted and analyzed. Once thawed, the samples should be processed immediately because freezing and thawing activates lipases in the milk (1). To repeat an often stated and apparently forgotten maxim, the analytical results are totally dependent upon the quality of the sample. All researchers who are planning to analyze human milk lipids must be certain that the samples they obtain are representative and defined.

ANALYTICAL RESULTS

Fatty Content

The report from Her Majesty's Stationery Office (7) contains possibly some of the most reliable data on the composition of human milk. Proper sampling was done and modern analytical techniques used. The report contains data on earlier work, most important nutrients including gross composition and cholesterol contents (Table I) as well as fatty acid identities. The average faty content given in this report was 4.2% which is higher than values in earlier and some recent papers, again suggesting improper sampling techniques.

[1]Scientific Contribution No. 786, Storrs Agricultural Experiment Station, University of Connecticut, Storrs, CT 06268.

Hambraeus (8) listed a content of 4.5% fat without any further descriptions of sampling. Belevady (9) found 3.2% ± 1.41 (mean ± SE) during the first month of lactation; 3.3% ± 1.2, 2-6 mo and 3.2% ± 1.3, 7-12 mo. Mellies et al. (10) obtained the following amounts after periods of dietary changes: maternal ad libitum, 3.58 ± 0.56 (mean ± SE); low cholesterol-high phytosterol, 2.69% ± 0.16 and the reverse of the low cholesterol-high phytosterol, 2.66% ± 0.16. Samples were obtained once/wk at the beginning or end of nursing by manual expression or use of a breast pump. Nayman et al. (11) have recently published the results of a comprehensive literature review on the composition of human milk in which they presented 4.12% ± 1.26 (SD) as a grand mean. Lauber and Reinhardt (12) detected quantities of 3.07% ± 0.65 (mean ± SD) in milk samples from 33 women of the Ivory Coast. The whole content of one breast was taken with an electrical pump. In the diet of the women, 75% of the calories were derived from carbohydrate, 10% from lipids and 15% from protein. The level of lipid remained relatively constant over 23 mo of lactation. Jelliffe and Jelliffe (13) compiled data on fat contents of mature human milk from well- and poorly nourished communities: the well-nourished fat content was 4.2 - 4.8%, and the poorly nourished, 2.0 - 4.4%. Total volumes diminished as the length of lactation progressed. Wurtman and Fernstrom (14) measured contents of 3.6% ± 0.3 (mean ± SEM) in the milk from 25 American mothers and 3.2 ± 0.4 from Guatemalan mothers who derived 80% of their calories from carbohydrates, primarily corn.

We obviously cannot overemphasize the importance of proper sampling, but would be remiss if we did not discuss extraction of the sample. The various groups used the following methods: (7) Roese-Gottlieb-diethyl ether, petroleum ether, ethanol; (10) Folch-$CHCL_3$-methanol; (12) modified Folch; (15) modified Roese-Gottlieb; Sanders et al. (16) used Folch, and Guthrie et al. (17) used diethyl-petroleum ether. In our opinion, the Bligh-Dyer method (18), a modified Folch extraction, is satisfactory and we recommend its use. The actual determinations have generally been done gravimetrically.

As a basis for comparison, we present some of the data from our earlier review (1). In Table II are possibly the only reliable data on the composition of the lipid classes of human milk lipids as represented by the fat globule figures. Incredibly, insofar as we know, there are no other recent publications on the subject but a phospholipid P content of 1.1 mg/100 ml of

TABLE I

Amounts of Water, Total Nitrogen[a], Protein[b], Fat, Carbohydrate[c], Energy[d], Nonprotein Nonamino Acid Nitrogen and Cholesterol in 100 ml of Pooled Mature Human Milk[e]

Center where obtained	Water	Total nitrogen	Protein	Fat	Carbohydrate	Energy (kcal)	Nonprotein nonamino acid nitrogen	Cholesterol
			(g)				(mg)	
Birmingham	90.1	0.19	0.95	3.9	7.3	66	44	16
Bristol	89.3	0.22	1.20	4.8	7.1	75	41	23
Cardiff	89.7	0.21	1.10	4.4	7.3	71	41	12
Edinburgh	90.1	0.21	1.00	3.7	7.3	65	51	20
Newcastle	89.3	0.22	1.10	4.2	7.8	71	55	10
Mean values	89.7	0.21	1.07	4.2	7.4	70	46	16

[a]Determined by Kjeldahl method.
[b]Calculated from total amino acid nitrogen (i.e., free amino acids plus those derived by hydrolysis of milk protein) multiplied by the factor 6.38.
[c]Expressed as monosaccharide.
[d]Calculated by applying the modified Atwater factors i.e., protein: 4 kcal/g; fat: 9 kcal/g: carbohydrate (as monosaccharide) 3.75 kcal/g.
[e]Adapted from Ref. 7.

TABLE II

Composition of Bovine and Human Fat Globule and Fat Globule Membrane Lipids[a]

Lipid	Bovine	Human		
		Fat globule	Fat globule membrane	
	(%)			
Hydrocarbons	tr	tr	tr	-
Sterol esters	tr	tr	tr	-
Triacylglycerols	97-98	98.1	58.2	60.0[b]
Diacylglycerols	0.28-0.59	0.7	8.1	-
Monoacylglycerols	0.016-0.038	tr	0.6	-
Free fatty acids	0.10-0.44	0.4	7.3	-
Sterols	0.22-0.41	0.248	0.653	5.6
Phospholipids	0.2-1.0	0.26	23.4	17.3

[a]Adapted from Ref. 1.
[b]Ref. 24.

milk has been reported (12). Data on bovine milk lipids are included for comparison. Note that at least 98% of the human milk lipids are triacylglycerols (TG). These overwhelming amounts cause difficulties in the separation and recovery of the other lipids, but separations can be attained by column and thin layer chromatography (GLC) (18-20).

The structure of the TG has been studied but not recently discussed (1). The structure is unique in that the *sn*-2 positions contain relatively large quantities of 16:0 as compared to most other food fats.

Sterols

We have not seen many publications on the contents of the other lipid classes in human milk with the exception of total phospholipids (12) and sterols. The amounts of total cholesterol in human milk have been reported as ranging from 200-564 mg/100 g of lipid (1). In more recent papers, the contents were: (7) 12-23 mg/100 ml of milk (See Table I), 240 ± 40 mg (mean ± SE)/100 g of fat (10), 11-23 mg/100 ml (11) and 12.6 ± 4.8 mg (mean ± SD) 100 ml milk (21). Data from the Picciano et al. study are in Tables III and IV. The most significant sources of variance in cholesterol content were: among-subject variability, 44% and among-day sampling, 33%. The authors recommended that a representative estimate of milk cholesterol content requires sampling from a large number of subjects throughout the day and over a period of days. Since cholesterol contents of fore and hind milks differed, it would be preferable again to take the entire contents from one breast. The authors observed that there was a significant correlation (Tables III and IV) between cholesterol and total lipid contents which, however, varied considerably in response to the circumstances of sampling.

Mellies et al. (10) investigated the effect of diets upon the cholesterol and phytosterol contents of milk. The results can be found in Table V. The cholesterol content did not respond to dietary manipulation, whereas phytosterols changed markedly, from 17 mg/100 g fat to 220 and then to 70 depending on the amount of phytosterol in the diet. The cholesterol and phytosterols, not further identified, were analyzed by gas liquid chromatography (GLC). It seems likely that some of the values for "total cholesterol" presented in earlier papers may have included phytosterol if the determinations were done colorimetrically. As shown in Table V, total sterols could include ca. 50% phytosterols if the diet is rich in these compounds and suitable analytical procedures should be employed. In addition, the individual phytosterols should be identified in order to determine if differential absorption and transfer occurs.

In contrast to the results already discussed, Tsang et al. (22) analyzed the milk from a woman with homozygous Type IIa hyperlipoproteinemia (high plasma cholesterol) and found a content of 650 mg/100 g of fat, as compared to 240 in milk from 14 normal women. The discriminatory process that maintained similar milk cholesterol levels with different diets (Table V and already mentioned) was apparently inoperative in the Type IIa disorder.

We still have not seen any comprehensive data on the fatty acid composition of the cholesteryl esters (1). Such information could provide insight on the transfer of these compounds from maternal blood to milk.

TABLE III

Means for Within-day and Weekly Cholesterol and Total Lipid Contents of Human Milk[a]

	Cholesterol, mg/100 ml				Fat, g/100 ml			Fat/ Cholesterol
	N	Mean[b]	SD	Range	Mean[c]	SD	Range	
Total	120	12.6	4.8	3.4-25.0	3.23	1.8	1.1-10.4	0.79
Within-day samples								
Early morning	18	15.7[d]	3.6		2.97[f]	1.9		0.93
Midday	18	15.1[d]	4.6		3.97[g]	1.3		0.55
Evening	18	17.8[e]	5.3		4.50[g]	2.2		0.43
Weekly samples[h]								
Week 1	22	11.3	4.6		2.86	1.6		0.86
Week 2	22	11.6	5.1		2.71	1.7		0.91
Week 3	22	11.1	3.8		2.69	1.4		0.79

[a]Adapted from Ref. 21.

[b]Means with unlike superscripts d and e differ $P < 0.01$.

[c]Means with unlike superscripts f and g differ $P < 0.05$.

[h]Early morning samples.

TABLE IV

Means for Cholesterol and Total Lipid Contents of Human Milk Representative of the Beginning and End of a Single Nursing[a]

	Cholesterol, mg/100 ml				Fat, g/100 ml			Fat/ Cholesterol
	N	Mean[b,c]	SD	Range	Mean[b]	SD	Range	
Totals	120	11.0	3.2	3.0-28.8	3.01	2.2	0.2-8.6	0.73
Foremilk								
Early morning	20	6.9[d]	3.8		1.46[d]	1.2		0.76
Midday	20	10.1[d,e]	3.4		2.99[e]	1.4		0.76
Evening	20	8.9[d,e]	3.3		2.72	1.5		0.69
Hindmilk								
Early morning	20	10.7[e]	5.2		2.86[e]	1.7		0.70
Midday	20	15.8[f]	5.9		4.02[e]	1.8		0.76
Evening	20	12.1[d]	5.6		3.67[e]	1.2		0.57

[a]Adapted from Ref. 21.

[b]Means with unlike superscripts d and e differ $P < 0.05$.

[c]Means with unlike superscript f differ, $P < 0.01$.

TABLE V

Effects of Dietary Cholesterol, Phytosterol and Polyunsaturate (P) Saturate (S) Ratio on Human Milk Sterols ($X \pm SE$)[a]

Milk Component	Maternal ad lib diet (P/S 0.53)	Low Cholesterol, high phytosterol diet (P/S 1.8)	High Cholesterol low phytosterol diet (P/S 0.12)
	(mg/100 g fat)		
Cholesterol	240 ± 40	240 ± 10	250 ± 20
Phytosterol	17 ± 3	220 ± 30	70 ± 10
Dietary cholesterol	450 ± 30	130 ± 5	460 ± 90
Dietary phytosterol	23 ± 8	790 ± 17	80 ± 1
Total fat	3.58 ± 0.56	2.69 ± 0.16	2.66 ± 0.16

[a]Adapted from Ref. 10.

To summarize our thoughts on the analysis of human milk sterols and steryl esters, we believe that: (a) a representative sample is required, (b) the amounts of cholesterol and phytosterol and their esters should be determined, preferably by GLC, (c) the fatty acids esterified to the sterols should be identified and (d) the various phytosterols should be separated and quantified.

Phospholipids

Data from our earlier review (1) on the composition of these lipids are in Table VI. When we wrote our review, there was little or no information on the glycosphingolipids, but recently some has been reported. Grimmprez and Montreuil (23) found ca. 40 mg of glycosphingolipid in the milk fat globule membranes (MFGM) from 1 ℓ of milk. Cerebrosides (glycosylceramides) and gangliosides were in the aqueous phase from a Folch extract of the MFGM and lactosylceramide in the solvent phase. Amounts were not given and the composition of the ceramide fatty acids was not determined.

Bouhours and Bouhours (24) analyzed the glycosphingolipids in the solvent phase from a Folch extract of MFGM. The lipid class composition is in Table II. The monohexosylceramides contained galactosyl (88%) and glucosylceramides (12%). A trace of lactosylceramide was observed. Hydroxy fatty acids (20% of total) were found in the galactosylceramides, but not in the glucosyl or lactosylceramides. The composition of the fatty acids will be given in Fatty Acids.

During milk secretion, the portion of the cell membranes which accompanies the milk fat globule when it is expelled from the cell is undoubtedly the source of the phospho- and glycosphingolipids in the MFGM (1). Because of their relative polarity, these compounds locate on the surface of the globule and contribute to emulsion stability. The phospholipids should be investigated more thoroughly because they contain relatively large quantities of long chain polyunsaturated fatty acids (1), to which Crawford et al. (25) have attributed a role in the development of the infant's brain and nervous system.

Fatty Acids

We compiled a list of the acids presumably identified in human milk (Table VII) in our review. Since then, several papers have been published which contain data on fatty acid composition. We have summarized most of these data in Table VIII. Minor acids (quantities less than 0.5%) have been omitted from the compilation. Note the remarkable similarity in fatty acid composition in fats from a very large number of samples, albeit some pooled, which would tend to smooth extremes. The only major variability is in 18:2, the content of which can be markedly altered by diet (1,6). We have added 16 acids to Table VII.

Even experienced investigators occasionally forget that GLC, although a superb method for resolution of fatty acid methyl esters, does not provide positive identification. Other pitfalls to be avoided are: quantitative credence given to small peaks on the recorder chart and not allowing enough time for the long chain polyunsaturated fatty acids to pass through the GLC column.

Hall (6) makes the important point that, except when bizarre diets are consumed, the fatty acid composition of human milk does not vary greatly. She found that the fatty acid composition of milk obtained from either breast (see also ref. 14) at the beginning or end of nursing and throughout 3 days of nursing (see also ref. 21) did not change. The stages of lactation and colostrum, for example, appar-

TABLE VI

Phospholipid Composition in Bovine and Human Milk Lipids[a]

Phospholipid mole%	Bovine	Human
Phosphatidylcholine	34.5	27.9
Phosphatidylethanolamine	31.8	25.9
Phosphatidylserine	3.1	5.8
Phosphatidylinositol	4.7	4.2
Sphingomyelin	25.2	31.3
Lysophosphatidylcholine	tr	1.4
Lysophosphatidylethanolamine	tr	3.7
Plasmalogens	3.0	
Diphosphatidylglycerol	tr	
Ceramides	tr	tr[b,c]
Cerebrosides	tr	tr[c]
Gangliosides	tr	tr[c]

[a]Adapted from Ref. 1.
[b]Adapted from Ref. 23.
[c]Adapted from Ref. 24.

TABLE VII

Fatty Acid Composition of Human Milk Lipids as of February 1980[a]

Number	Type	Identity
	Saturates	
10	normal, even	4:0-22:0
7	normal, odd	11:0-23:0
49	monobranched	10:0-18:0[b]
5	multibranched	12:0-13:0 15:0-16:0[c]
	Monoenes	
63	*cis*[d]	10:1-18:1,20:1, 23:1-24:1-26:1[e] 23:1
3	*trans*	16:1,18:1 20:1
22	Dienes	12:2-22:2 all even *cis, cis,*; *cis, trans*; *trans, trans*; positional isomers
	Polyenes	
7	tri	18:3,20:3,[f]22:3 geometric and positional isomers
3	tetra	20:4,[f]22:4
3	penta	20:5,22:5[f]
1	hexa	22:6
1	Cyclic hexane	11, terminal hexane
9	Hydroxy	16:0,18:0,20:0,22:0[e] 23:0,24:0,24:1,25:0 26:0
Total 183		

[a]Adapted from Ref. 1.
[b]Signifies n-acids with methyl branches.
[c]Signifies n-acids with 3 or 4 methyl branches.
[d]Designated as *cis*, but not usually determined.
[e]Ref. 24.
[f]Ref. 31: 20:3ω3 or ω6; 20:4ω3 or ω6 and 22:5ω3 or ω6.

TABLE VIII

Fatty Acid Compositions of Human Milk Fat (wt %)[a]

Fatty Acid	Reference								
	26[b]	9[c]	8[d]	7[e]	14[f]	17[g]	11[h]	30[i]	6[j]
	Mean ± SD	Mean ± SE			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SE	Mean ± SE
12:0	4.6 ± 0.55	3.5 ± 3.56	4.8	5.4	5.0 ± 0.8	3.8 ± 1.6	6.3 ± 2.6	6.8 ± 0.7	5.60 ± 0.27
14:0	6.4 ± 0.11	11.1 ± 4.21	6.2	7.3	6.1 ± 1.2	5.2 ± 1.7	8.6 ± 2.5	11.0 ± 1.1	9.80 ± 0.12
16:0	23.4 ± 0.67	23.5 ± 3.00	23.7	26.5	24.7 ± 2.7	22.5 ± 2.6	21.0 ± 2.7	25.0 ± 0.6	24.46 ± 0.47
16:1	3.7 ± 0.29	2.7 ± 2.01	4.6	4.0	4.1 ± 0.6	4.1 ± 2.3	-	1.9 ± 0.2	- 0.28
18:0	8.6 ± 0.61	4.4 ± 1.88	6.7	9.5	8.7 ± 1.0	8.7 ± 1.4	7.0 ± 1.2	6.4 ± 0.3	9.72 ± 0.28
18:1	33.3 ± 1.66	35.3 ± 8.11	37.4	35.5	34.1 ± 2.1	39.5 ± 3.8	37.3 ± 4.3	32.0 ± 1.1	32.84 ± 0.50
18:2	12.0 ± 1.32	14.4 ± 8.62	9.0	7.2	13.0 ± 2.9	14.4 ± 4.1	10.0 ± 2.2	9.1 ± 0.5	7.47 ± 0.44
18:3	1.8 ± 0.29	-	3.4	0.8	-	2.0 ± 0.7	1.0 ± 0.5	0.8 ± 0.1	0.67 ± 0.08
20: Polyene	0.9 ± 0.11	-	-	>0.5	-	-	2.9 ± 0.15	2.4	2.37

[a]Trace amounts of fatty acids less than 0.5% have been omitted.

[b]Pooled samples from 15-20 donors.

[c,d]Number of donors not reported.

[e]Pooled samples from 96 donors; complete contents from one breast.

[f]Data presented are the fatty acids in the milk from the left breast of one donor.

[g]Forty ml samples provided by 25 donors.

[h]Compiled from earlier references. The 20:polyene figure includes 22:polyenes.

[i]Total number of samples, 231 from 4 countries, sampled at 4-6 wk post partum. Data are weighted.

[j]Mean composition of milk collected from one donor 4 times during 3 consecutive days.

ently do not influence the fatty acid composition (26). We must remember, however, that the absolute concentrations of lipid classes could change during any of the circumstances already listed and these possibilities have not been investigated.

Specifically, the *trans* fatty acids and long chain polyunsaturates have been analyzed. We listed *trans* isomer contents of 2-18% of milk fatty acids in our review (1). These isomers most certainly are derived from the consumption of partially hydrogenated food fats by the mother who is breast-feeding. Both geometric and positional isomers of unsaturated fatty acids have been identified in, for example, margarines (27).

Beare-Rodgers et al. (28) determined that most of the polyenoic acids in human milk reacted with lipoxidase and thus contained the *cis,cis*-methylene interrupted series of double bonds. The *trans* isomers were found primarily in the 18:1 fraction. Therefore, we can expect that the 18:2 content as determined by GLC consists mostly of 9,12-*cis,cis*-18:2, the major dietary essential fatty acid. The relatively new, highly polar GLC column packings such as SP 2330 (Supelco, Inc., Bellefonte, PA) can provide hitherto unattainable separations.

The effects of diet have been further investigated. Sanders et al. (29) obtained the data in Table IX on the milk from total vegans who do not eat foods from animal sources and from omnivore controls. Identifications were made by comparisons of retention times to known standards and carbon number plots. The milk from the omnivores contained more saturated and less unsaturated fatty acids than the samples from the vegans. Also notable is the difference in 18:2 content, 6.9 vs 31.7%. Unfortunately, the authors did not determine the sterol contents of the milks. In the FAO publication (30), contents of long chain essential fatty acids were reported as follows: $\omega 6$; 20:3 + 20:4 + 22:4 + 22:5, 1.0 ± 0.3 (mean ± SE) and $\omega 3$; 20:5 + 22:6; 1.4 ± 0.1. Sanders and Naismith (31) provided more data on the content (wt % of total acids) of $\omega 3$ and $\omega 6$ polyunsaturated fatty acids and these are in Table X. In several of the acids, the location of the double bonds has probably not been ascertained. These are: $\omega 3$; 20:3, 20:4, 20:5 and 22:5 $\omega 3$ or $\omega 6$. For comparison, the composition of a milk formula is included.

Mellies et al. (32) obtained the data in Table XI which illustrate the influence of dietary manipulation on fatty acid composition. The ad libitum diet was that consumed by the subjects prior to changes in diet. The P (polyunsaturate)-rich diet was similar to the National Heart Lung and Blood Institute Type II diet with a p/s (saturate) ratio of 1.8. The s/ (saturate) rich diet and a p/s ratio of 0.12 and was designed to be typically American. Again, the increase in 18:2 in the p group was reciprocated by a decrease in the amount of 16:0.

Lauber and Reinhardt (12) analyzed the milk from 33 women living on the Ivory Coast once/mo for 23 mo. Their data for both TG and phospholipid fatty acids are in Table XII. The diets were high in carbohydrate and low in both protein and essential fatty acids. The amount of 18:2 in the milk, 5.7%, is ca. one-half of the content found in the milk from women identified as consuming an adequate diet (1). The total phospholipids (1.11 mg P/100 ml), not unexpectedly, contained more long chain polyunsaturated fatty acids than the TG.

Bouhours and Bouhours (24) characterized by GLC the fatty acids in MFGM, monohexosylceramide and lactosylceramides. The data are presented in Table XIII. The hydroxy fatty acids have not been previously found in human milk lipids, nor have 23:1, 24:1, 25:1 and 26:1. Their presence, so far only in the monohexosylceramides, poses questions as to their origin and metabolism in relation to milk secretion and MFGM function.

We should, however, repeat the observations of Hall (6) concerning the uniformity of human milk. She correctly noted that the composition shows limited changes when the diet is nutritionally adequate. Variations resulting from malnutrition, undernutrition or supplementation are not random and can be predicted.

The composition of milk from mothers of premature babies has not been even cursorily investigated and should be because it is banked and used to feed the infants. We know virtually nothing about the lipid composition therein (33).

In summary, we nevertheless believe that research on human milk lipids has been neglected. In this review, we have indicated many aspects which should be investigated. While it is difficult to obtain proper samples, the major problem seems to be one of attitude; human milk is not considered a food.

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TABLE IX

Breast-milk Fatty Acids in Vegans and Omnivore Controls[a]

Methyl esters	Vegans		Controls	
	Mean	SE	Mean	SE
12:0	39	12.5	33	7.1
14:0	68	17.0	80	5.3
16:0	166[b]	14.4	276	10.5
18:0	52[b]	5.9	108	8.5
16:1	12[b]	0.9	36	5.3
18:1	313	25.0	353	11.1
18:2ω6	317[b]	44.5	69	8.1
18:3ω3	15[b]	2.4	8	0.5
20:2ω6	5.1[b]	0.7	1.8	0.3
20:3ω6	3.1	0.8	2.3	0.2
20:4ω6	7.2	2.1	5.4	1.3
22:4ω6	1.4	0.5	0.8	0.3
22:5ω6	0.9	0.4	1.5	1.2
20:4ω3	0.3[b]	0.1	0.9	0.2
20:5ω3	0.4	0.1	2.0	0.8
22:5ω3	2.7	1.4	5.2	2.7
22:6ω3	2.3	1.1	5.9	2.3

[a]Mean values expressed as mg/g total methyl esters for 4 vegans and 4 omnivore controls. Vegans do not eat foods from animal sources (Ref. 29).

[b]Statistical significance of difference between means shown when $P < 0.05$.

TABLE X

Polyunsaturated Fatty Acids Provided by a Cow's Milk Formula and by Breast-milk[a]

Fatty acid methyl esters	Milk formula	Breast-milk (4 samples)	
		Mean	SE
	(wt. %)		
18:2ω6	1.60	6.90	0.81
18:3ω3	0.70	0.80	0.05
20:2ω6	nd[b]	0.18	0.03
20:3ω6	0.06	0.23	0.02
20:4ω6 + 20:3ω3	0.10[c]	0.54[d]	0.01
20:4ω3	0.06	0.09	0.02
20:5ω3	0.08	0.20	0.08
22:4ω6	0.01	0.08	0.03
22:5ω6	0.01	0.15	0.12
22:5ω3	0.11	0.52	0.27
22:6ω3	0.02	0.59	0.23

[a]Ref. 31.

[b]nd, not detected.

[c]Mainly 20:3ω3.

[d]Mainly 20:4ω6.

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TABLE XI

Maternal Milk Fatty Acids (g/100 g fatty acid) during ad libitum Diet, Polyunsaturate-rich Diet (P) and Saturate-rich Diet (S), (X ± SE)[a,b]

Fatty acid	Ad libitum	P-Rich	S-Rich
14:0	5.8 ± 0.5	4.7 ± 0.5[e]	7.2 ± 0.4
16:0	21.9 ± 0.8[i]	18.8 ± 0.7[f]	23.8 ± 0.5
16:1	3.8 ± 0.4[g]	2.7 ± 0.2[c]	3.7 ± 0.2
18:0	7.6 ± 0.3[g]	6.4 ± 0.3[f]	8.6 ± 0.3
18:1	37.7 ± 0.5[g]	34.8 ± 1.1	36.7 ± 0.6
18:2	14.5 ± 1.4[j]	24.4 ± 1.9[f]	10.1 ± 0.6
20:0	0.2 ± 0.02	0.24 ± 0.02	0.21 ± 0.01
18:3	1.9 ± 0.1[j]	1.4 ± 0.04	1.6 ± 0.05[k]
20:3	0.53 ± 0.08	0.59 ± 0.03[e]	0.30 ± 0.05[k]
20:4	0.56 ± 0.03[g]	0.45 ± 0.03	0.47 ± 0.03

[a]Ref. 32.

[b]P-rich compared to S-rich: [c]$P < 0.05$, [d]$P < 0.02$, [e]$P < 0.01$, [f]$P < 0.001$.
P-rich compared to ad libitum: [g]$P < 0.05$, [h]$P < 0.02$, [i]$P < 0.01$, [j]$P < 0.001$.
S-rich compared to ad libitum: [k]$P < 0.05$.

TABLE XII

Percentage of the Fatty Acids in Triglycerides and Phospholipids of Breast Milk from Ivory Coast Donors[a]

	Mean ± SD	Mean ± SD
8:0	0.11 ± 0.04	
10:0	1.39 ± 0.33	0.54 ± 0.20
12:0	9.31 ± 1.97	4.52 ± 1.00
14:0	15:20 ± 3.30	15.20 ± 2.40
15:0	0.47 ± 0.11	0.68 ± 0.15
16:0	27.90 ± 2.20	35.90 ± 4.60
16:1	3.23 ± 0.60	3.87 ± 0.75
17:0	0.77 ± 0.15	0.93 ± 0.17
17:1	0 47 ± 0.12	0.63 ± 0.16
18:0	6.92 ± 1.32	7.20 ± 1.10
18:1	25.40 ± 3.90	15.50 ± 2.70
18:2	5.70 ± 1.10	5.35 ± 0.79
18:3	0.29 ± 0.08	0.39 ± 0.16
20:0	0 23 ± 0.10	0.42 ± 0.20
20:4	0.31 ± 0.10	1.06 ± 0.32

[a]Adapted from Ref. 12.

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TABLE XIII

Fatty Acid Composition of Monohexosylceramide and Lactosylceramide of Human Milk Fat Globule Membrane[a]

Fatty acid methyl esters	Monohexosylceramide		Lactosylceramide
	NFA[b]	HFA[c]	NFA
	(wt %)		
16:0	13.6	3.9	16.2
16:1	1.3	-	0.9
18:0	6.9	2.9	8.7
18:1	5.2	-	7.8
20:0	3.8	1.2	2.8
20:1	0.3	-	0.2
22:0	13.3	19.9	12.5
23:0	3.9	10.6	3.4
23:1	0.4	-	1.1
24:0	31.9	51.9	20.1
24:1	16.8	5.0	20.1
25:0	0.3	1.1	1.7
25:1	0.8	-	2.6
26:0	0.7	3.5	0.6
26:1	0.8	-	1.3

[a]Ref. 24.

[b]NFA: nonhydroxy fatty acids.

[c]HFA; hydroxy fatty acids.

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The Fatty Acid Composition of Three Unicellular Algal Species Used As Food Sources for Larvae of the American Oyster (*Crassostrea virginica*)[1]

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ABSTRACT

The total lipid and fatty acid content of 3 algal species, *Pyramimonas virginica*, *Pseudoisochrysis paradoxa* and *Chlorella* sp., which have been successful as food sources for rearing larvae of the American oyster, *Crassostrea virginica*, was determined. Of the fatty acids of $\omega 6$ and $\omega 3$ families which have been shown to be essential fatty acids for normal growth in many animals, only the $\omega 6$ fatty acids were found to be higher in these 3 species of algae than in the traditional oyster larvae diet which consists of the algae *Monochrysis lutheri* and *Isochrysis galbana*. The major fatty acid constituents of the total lipids of the 3 species were the C12, C14, C16 and C18 saturated fatty acids and the C16 and C18 mono- and polyunsaturated acids. These components constituted 70-93% of the total lipid in cultures of all ages. There were modest amounts of C20 and C22 polyunsaturated acids; some of these existed only in trace amounts. In *P. virginica* and *Chlorella* sp., hexadecanoic acid was dominant (23-39%). The presence of large quantities of tetradecanoic acid (22-26%) and oleic acid (17-21%) was characteristic of *P. paradoxa*. *Chlorella* sp. had the highest proportion of octadecatrienoic acid (18:3ω3) which accounted for up to 17% of the total lipids. γ-Linolenic acid (18:3ω6) was found only in *Chlorella* sp., but in the 5th-day culture only. The lowest proportion of total polyethylenic acid was in *P. paradoxa*; however, lipid analyses showed this alga had the most lipid/individual cell. Some variations were observed in the fatty acid composition with age of the culture.

INTRODUCTION

In the past few decades, a number of studies have examined the nutritional values of different phytoplankton species as food for molluscan bivalve adults and larvae (1-10). All these studies indicated that diets composed of 3-4 species of algae generally support faster growth than diest consisting of only 1 or 2 species. In the Virginia Institute of Marine Science (VIMS) marine culture laboratory, a combination of 4 algal species, *Nannochloris oculata*, *Chlorella* sp., *Pyramimonas virginica* and *Pseudoisochrysis paradoxa* has been successfully used as a standard diet for oyster larvae culture (10,11).

Windsor (12) fed the algal species *P. paradoxa*, *P. virginica*, *Chlorella* sp., and *N. oculata* to oyster larvae *(Crassostrea virginica)* singly and in various combinations. The results were then compared with larvae fed with *Monochrysis lutheri* and *Isochrysis galbana*, which are the traditional diets for American oyster larvae, and indicated that *P. virginica* was the most influential dietary component. The best diet, judged in terms of maximal growth rate, survival, pediveliger production and setting success, was a combination of *P. paradoxa*, *P. virginica* and *Chlorella* sp. The best diet was labeled as "good food" whereas the rest were labeled as "mediocre food."

Aside from the biological approach, there have been very few qualitative and quantitative analyses of the major chemical components (protein, lipid and carbohydrates) of algal species used as larval oyster foods. Most nutritional studies of oysters have been performed only on the adult stage (13-17). Only 2 papers (18,19) have dealt with the needs of larvae during development, metamorphosis and early spat growth.

By culturing *Thalassiosira pseudonana* under light of different spectral distribution and by harvesting this algal culture from either exponential or stationary phases, Flaak and Epifanio (13) successfully produced 6 diets of differing carbon-to-nitrogen ratios as feed for oysters. They concluded that oysters grew more rapidly when fed with diets richer in carbohydrates than in proteins and that in the adult oyster, polysaccharides have been found to serve as a major energy reserve (20). Castell and Trider (14) fed oysters with formulated feeds of varying protein-carbohydrate ratios and observed that diets with a 60% carbohydrate content gave rise to higher glycogen production in oysters than diets with a 20% carbohydrate content, supporting previous oyster nutrition studies (15-17).

In view of the results of nutritional studies in both adult and larval oysters, it seems likely that carbohydrate is important for

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growth and accumulation of glycogen in juvenile and adult oysters, whereas lipid could play a vital role in development and metamorphosis in oyster larvae. This paper describes the results of analyses for total lipids and fatty acid compositions of *P. virginica*, *P. paradoxa* and *Chlorella* sp. and compares the results of lipid analyses of *M. lutheri* and *I. galbana* reported by Ackman et al. (21), Watanabe and Ackman (22), Chuecas and Riley (23) and Joseph (personal communication). According to Otsuka and Morimura (24), fatty acid compositions of *Chlorela ellipsoidea* cultures changed with age. Ackman et al. (25) also observed changes in the relative proportions of fatty acids, including shifts in the biosynthetic pathway for acids belonging to either the linoleic or linolenic systems. Therefore, the fatty acid composition of algal cultures of different ages were examined, although algal cells used as larval food are harvested only during the log growth phase (10-15 days).

MATERIALS AND METHODS

Since it is not economical to grow axenic algal mass cultures, all the algal species used as food to rear oyster larvae in the marine culture laboratory of VIMS are produced in mass cultures which are not axenic. The algal cultures used for fatty acid analysis were grown under the same conditions as the mass cultures except that the scale was much smaller.

The 3 algal species were cultured at 16-19 C in 3-ℓ fernbach flasks containing 1.5-2.0 ℓ pasteurized and filtered estuarine water at 13-20% salinity, enriched with N_2M Medium (nutrient enriched medium developed at VIMS), Guillard's vitamin mix and soil extract (26). *P. virginica* and *P. paradoxa* were grown using a continuous light source (2000 lux); *Chlorella* sp. was grown at 3,300 lux. A current of air provided circulation and aeration. *P. virginica* is a flagellated unicellular alga of the class Prasinophyceae, *P. paradoxa* belongs to the class Haptophyceae and *Chlorella* sp. is a representative of the class Chlorophyceae.

Three separate batches of culture of each species were grown for fatty acid analyses. Algal cells were harvested by centrifugation and filtration on the 5th, 10th, 15th and 20th days of culture. Cell counts of algal samples were made at the time of harvesting. The algal pellets were drained to determine approximate cell wet weight. The Bligh and Dyer method (27) was employed to extract the total lipids of the pelleted cells. Lipids were subsequently transesterified with BF_3 in CH_3OH (14%, v/v) with additional CH_3OH and benzene (28).

Fatty acid methyl esters (FAME) of the algae were chromatographed on a Varian Model 3700 gas chromatograph equipped with dual hydrogen flame ionization detectors and a 2-channel Omniscribe strip chart recorder. Both polar and nonpolar columns (8 ft, 4 mm id glass) were used to separate the algal FAME; these columns were 3% EGSP-Z (100/120 mesh, gas-chrom Q) and 3% SE-30 (gas-chrom Q, 100/120 mesh), respectively. Following a 3-min hold at initial temperature, the columns were temperature-programmed from 100-170 C at 8 C/min. The flow rates of nitrogen, compressed air and hydrogen were 40 ml/min, 300 ml/min and 30 ml/min, respectively.

Tentative identification of FAME was based on comparisons with chromatograms of commercial fatty acid standards and with the methyl esters of cod liver oil, which were used as a secondary reference standard (29) and analyzed under the same chromatographic conditions. Semilog plots of $^{r}18{:}0$ (retention time relative to 18:0) against the carbon chain length were constructed for the algal total lipid FAME and standards to aid identification (30). Subsequently, the Appelqvist method of hydrogenation (31) was also performed on the algal FAME mixture to confirm the accuracy of identification and quantification of major components. The chromatograms were quantitated by triangulation. The estimation of the total lipid in wet algal cells was based on the sulfophosphovanillin method (32).

RESULTS AND DISCUSSION

Cell counts for the 3 species studied are given in Table I. Algal cultures usually reached log phase in 10-15 days. The death phase of *P. virginica* came earlier. After the 15th day, the number of cell count dropped. Interesting changes were observed in the total monoethylenic and polyethylenic acids. The total monoethylenic acid increased whereas total polyethylenic acid decreased on the 20th day. The results of the estimation of total lipid in terms of μg lipid $X10^{-7}$/cell and ratio of μg lipid-to-μm^3 cell volume are also given in Table I. *P. paradoxa* showed a greater amount of total lipid/cell than the other 2 species. The yields of wet cell weights of algae in all our experiments were inconsistent; therefore, weight data were not used to calculate the content of lipid/mg wet algae.

The results obtained from the analyses of fatty acid composition of 3 species of algae at different culture ages are shown in Tables II-IV. The major fatty acid constituents in the total lipids were the saturated fatty acids of the even

TABLE I

Comparison of Cell Density to Age of Culture and Total Lipid Content at 15 Days for the Algal Species Used As Food for Oyster Larvae

	Algal Species		
	P. paradoxa	*P. virginica*	*Chlorella* sp.
	(Cell counts per ml x 10^6)		
Day after inoculation			
5	6.1	2.7	14
10	9.8	11.o	68
15	18.0	11.0	130
20	19.0	5.0	190
μg Lipid x 10^{-7}/cell	22.91	15.22	1.48
Mean individual cell volume (μm^3)	47.70	33.50	4.85
μg Lipid x $10^{-7}/\mu m^3$ cell volume	.48	.45	3.1

chain lengths C12-C18, and mono- and poly-unsaturated C16 and C18 acids. These components comprised 70-93% of the total lipids in all ages, whereas odd-numbered saturated and other saturated fatty acids accounted for 3-18%. There were modest-to-trace amounts of polyenic acids of the C20 and C22 chain length.

The fatty acid spectra of these 3 algal species were qualitatively similar and the major fatty acids were those found by earlier investigators (21,23,26,33). In *P. virginica* and *Chlorella* sp., hexadecanoic acid (16:0) was dominant (23-39%). This result is similar to the findings of Ackman et al. (21), (22), Chuecas, Watanabe and Cakman and Riley (23) and Kates and Volcani (33). The presence of large quantities of tetradecanoic acid and oleic acid (22-26% and 17-21%, respectively) was characteristic of *P. paradoxa*. Tetradecanoic acid has also been reported as a prominent fatty acid in the Chrysophyceae, *M. lutheri* (21,23) and *I. galbana* (22), but the level was much lower (9-11%). Among the 3 algal species tested, *Chlorella* sp. had the highest proportion of α-linolenic acid (18:3ω3) (17% of the total fatty acids in the 10th day culture) whereas γ-linolenic acid (18:3ω6) was found only in *Chlorella* sp. and then only in the 5th day culture. This suggests that the series of reactions, 18:2ω6$\rightarrow$18:3ω6$\rightarrow$20:4ω6 might be active only up to the 5th day in this species. Comparatively higher levels of 22:6ω3 were also observed in *P. virginica* and *P. paradoxa*. The presence of iso and anteiso fatty acids suggests contamination by bacteria (34,35) since the algal cultures were not bacteria-free.

There were some variations in the proportion of certain fatty acid components during the growth period (Tables II-IV). Therefore, the culture age of algae may affect the synthesis of certain fatty acids (24,25). For example, in *Chlorella* sp., the proportion of 12:0 was highest in 5th day cultures whereas 16:1 disappeared on the 20th day. In contrast, *P. virginica* had the highest proportion of 12:0 and 16:1 on the 20th day. The proportion of 18:1 in latter stages (20th day) was highest in all 3 species. The effect of age was also observed in the relative increase of total C18 acids in the 3 species; this effect could possibly have resulted from the accumulation of reserve fat for cell division (24). However, in *Chlorella* sp., the pattern of increase was not linear in the total C18 acids, which showed a decrease on the 15th day and an increase on the 20th day culture.

Differences in weight percentages of fatty acids were also observed in different cultures of the same species. Many factors could have caused these differences even though cultures were grown under the same conditions. The chemical composition of the sea water is almost constant; however, the relative concentration of salts varies, depending on weather conditions and season. Similarly, the concentration of various chemicals in soil extract probably varied from location to location. Several investigators (36-40) reported the fatty acid composition of algae may be altered by variables such as the level of available nitrate.

Data from the analyses of the fatty acid compositions of these 3 algal species judged to be "good food" for oyster larvae did not show appreciable qualitative differences from the data on species which have been judged to be "mediocre food" (*M. lutheri* and *I. galbana*) as shown by Joseph (personal communication) and other investigators (21-23). Their results are tabulated in Table V for comparison. In particular, the 15th day cultures of *M. lutheri*

TABLE II

Fatty Acid Weight Percent Composition of *Pyramimonas virginica*

Fatty acid	Culture day			
	5	10	15	20
Saturated				
12:0	2.40	3.60	3.45	8.70
13:0	1.15	0.60	1.30	1.30
14:0	4.30	2.60	2.00	4.95
15:0	0.86	0.45	0.45	-
16:0	29.10	34.25	31.40	23.30
17:0 ± 16:2	2.30	2.06	1.20	3.75
18:0	4.05	2.80	4.20	3.80
Total	44.16	46.35	44.00	45.80
Monoethylenic				
14:1ω5	2.10	1.65	4.15	6.90
16:1	2.60	2.00	2.50	5.75
18:1	3.10	3.10	3.90	15.70
20:1	-	Tr[a]	Tr	Tr
22:1	0.20	0.30	-	-
Total	8.00	7.05	10.55	28.35
Polyethylenic				
16:3ω3	8.20	10.30	9.60	5.78
18:2ω6	3.35	2.55	2.00	0.80
18:3ω3	5.85	7.50	5.85	1.90
18:4ω3	4.95	5.10	6.45	3.90
20:2ω6	2.65	2.75	2.85	0.70
20:3ω3	-	0.20	0	0
20:4ω6	0.85	3.35	0.80	8.60
20:4ω3	-	-	-	-
20:5ω3	1.55	-	2.50	-
20:5ω3	Tr	0.90	0.95	0.50
22:6ω3	2.60	3.05	2.75	1.40
Total	30.00	35.70	33.75	23.58
Other saturated				
7:0	3.90	0.85	2.10	2.85
8:0	0.45	-	-	-
10:0	1.10	0.25	0.45	-
11:0	2.50	1.05	0.85	1.20
14:0 iso	1.40	0.65	0.65	2.05
15:0 anteiso	0.80	0.45	0.45	-
16:0 iso	0.30	0.45	0.20	-
18:0 iso	2.70	1.50	1.30	-
Total	13.15	5.20	6.00	6.10
Unknowns total	1.55	1.85	2.50	1.20

[a]Trace (less than 0.04%).

and *I. galbana*, which were analyzed by Joseph, were supplied by our laboratory and were cultured under similar conditions. The results indicated that the amount of linoleic family (ω6) fatty acid was higher in the "good food" algae than in the "mediocre food" algae. The amount of ω3 fatty acid was similar in both the "good" and "mediocre" algae except for *P. paradoxa*, which was lower (Table VI). The amount of total polyethylenic acids of *M. lutheri* reported by Ackman and Chuecas was higher; this may have resulted from differences in growth conditions and harvesting times.

Fatty acids of the ω6 and ω3 families have been shown to be essential in many animals. Some mammalian species such as the rat have a high requirement for ω6 fatty acids and a low requirement for ω3 fatty acids (41). For the rainbow trout, the situation is reversed—ω3 fatty acids are the most important (42,43). However, German carp apparently require both

TABLE III

Fatty Acid Weight Percent Composition of *Chlorella* sp.

Fatty acid	Culture day 5	10	15	20
Saturated				
12:0	7.82	3.23	2.75	2.75
13:0	1.08	1.28	1.55	0.15
14:0	5.37	2.48	2.35	0.95
15:0	0.17	Tr[a]	0.70	Tr
16:0	26.68	27.23	27.00	39.45
17:0 ± 16:2	1.10	5.28	3.05	3.60
18:0	3.45	1.37	1.45	2.35
Total	45.67	40.87	38.95	49.25
Monoethylenic				
14:1ω5	0.63	Tr	0.30	0.10
16:1	2.70	3.65	5.15	-
18:1	9.18	7.57	7.20	15.00
20:1	0.57	0.30	-	0.95
22:1	Tr	Tr	6.40	-
Total	13.08	11.52	19.05	16.05
Polyethylenic				
16:3ω3	2.25	6.47	4.90	3.85
18:2ω6	6.98	11.03	7.55	14.20
18:3ω6	0.67	-	-	-
18:3ω3	12.70	16.65	12.40	10.45
18:4ω3	0.20	0.60	1.50	0.40
20:2ω6	0.17	Tr	1.00	-
20:3ω3	-	-	0.20	-
20:4ω6	0.80	0.40	0	-
20:4ω3	Tr	Tr	0.85	-
20:5ω3	0.17	0.17	0.10	0.50
22:4ω3	0.17	-	-	-
22:5ω6	-	-	0.35	-
22:5ω3	-	Tr	0.25	-
22:6ω3	Tr	Tr	0.60	-
Total	24.11	35.32	29.70	29.40
Other saturated				
7:0	0.77	0.30	1.25	1.80
9:0	0.70	0.20	0.80	0.80
10:0	0.67	-	0.10	Tr
11:0	4.30	2.03	2.30	1.90
14:0 iso	1.37	1.87	1.55	0.15
15:0 anteiso	0.45	-	0.68	Tr
16:0 iso	1.10	Tr	0.70	0.80
18:0 iso	2.27	0.70	0.75	-
Total	11.63	5.10	8.13	5.45
Unknowns total	3.93	3.28	2.35	1.26

[a]Trace (less than 0.04%).

ω6 and ω3 in the diet (44). Similar requirements could probably exist for the growth and metamorphosis of oyster larvae from free swimming form to settled spat. The high ratios of ω6/ω3 in our results (Table VI) strongly suggest that ω3 and ω6 fatty acids are equally important for the growth and development of oyster larvae. It is notable that for the adult *C. virginica*, the ratio ω6/ω3 calculated from the data of Watanabe and Ackman (22) is low (0.16). It is possible that the nutritional requirements for the growth of oysters and oyster larvae are different. The chemical components which are considered essential for adult oysters might not be as important for the larvae. For example, the results of previous nutritional studies of the adult oyster (14-17) showed that carbohydrate was important for the growth of

TABLE IV

Fatty Acid Weight Percent Composition of *Pseudoisochrysis paradoxa*

Fatty acid	Culture day 5	10	15	20
Saturated				
12:0	4.10	2.80	0.90	2.65
13:0	0.97	0.80	0.45	0.20
14:0	26.00	23.77	23.10	22.10
15:0	0.47	0.47	0.20	0.15
16:0	17.53	18.40	15.65	18.25
17:0 ± 16:2	1.45	0.67	0.25	0.50
18:0	1.40	1.47	0.95	1.10
Total	51.92	48.38	41.50	44.95
Monoethylenic				
14:1ω5	1.00	0.63	1.30	Tr[a]
16:1	3.87	4.77	3.65	3.40
18:1	16.90	18.13	18.20	21.25
20:1	0.97	0.63	0.95	0.95
22:1	0.30	0.27	0.20	-
Total	23.04	24.43	24.30	25.60
Polyethylenic				
16:3ω3	1.97	1.27	1.05	1.40
18:2ω6	3.27	4.23	3.00	3.85
18:3ω3	1.90	2.13	2.00	2.20
18:4ω3	1.40	1.63	2.20	2.20
20:2ω6	0.57	0.73	0.35	0.65
20:3ω3	0.30	0.27	-	Tr
20:4ω6	0.10	0.17	Tr	Tr
20:4ω3	0.07	-	Tr	Tr
20:5ω3	0.20	0.40	0.05	0.10
22:5ω3	0.47	-	0.35	0.30
22:6ω3	1.87	3.10	2.20	2.05
Total	12.21	13.93	11.20	12.75
Other saturated				
7:0	0.97	-	0.80	1.15
9:0	0.30	-	0.10	0.10
10:0	0.13	-	Tr	0.15
11:0	0.57	0.60	0.15	0.15
14:0 iso	-	-	0.45	0.20
15:0 anteiso	0.17	0.10	0.10	0.10
16:0 iso	0.67	0.33	0.15	Tr
18:0 iso	1.97	1.97	-	0.50
Total	4.78	3.00	1.75	2.35
Unknowns totals	3.48	7.26	19.85	14.25

[a]Trace (less than 0.04%).

oysters and was the major reserve of energy. Flaak and Epifanio (13) reported that *T. pseudonana* cells with relatively high carbohydrate content have greater nutritional value for oysters and that the total protein requirements of oysters is relatively low. However, studies on the biochemical changes in *O. edulis* larvae by Holland and Spencer (18) indicated the developing larvae accumulated neutral lipid which increased from 8.8% of the total organic matter (dry wt basis) in newly released larvae to 23.2% at metamorphosis. During metamorphosis, neutral lipid decreased to 9.6% of the total organic matter and then remained constant up to 25 days after settlement. Additional studies on *O. edulis* larvae by other investigators showed that lipid was not only the major energy reserve during growth but also during starvation periods, (19,45) and that the viability of larvae was unrelated to the initial glycogen content (46).

The results of our studies on the fatty acid composition of the 3 algal species used as food for oyster larvae show that the fatty acid

TABLE V

Fatty Acid Weight Percent Composition of *M. lutheri* and *I. galbana*

Algal species (References)	*M. lutheri* (21)	*M. lutheri* (23)	*M. lutheri* (Joseph: personal communication)	*I. galbana* (Joseph: personal communication)	*I. galbana* (22)
Fatty acid	Weight percent composition				
Saturated					
12:0	0.2	0.6	1.2	2.9	0.6
13:0	-	0.2	0.7	0.7	0.2
14:0	11.2	9.2	13.8	9.7	10.6
15:0	0.2	0.4	0.9	1.2	1.3
16:0	15.1	10.1	28.9	21.3	22.0
17:0	-	0.4	-	-	-
18:0	-	0.4	0.8	2.5	2.3
20:0	-	-	0.7	Tr	-
Total	26.5	21.3	47.0	38.3	37.0
Monoethylenic					
14:1	0.2	-	Tr	0.7	-
16:1	25.4	20.2	21.2	21.7	15.7
17:1	-	-	0.9	3.2	-
18:1	3.4	5.7	7.2	6.3	13.5
20:1	0.6	-	-	-	1.2
Total	29.6	25.9	29.3	31.9	30.4
Polyethylenic					
16:2ω7	0.2	2.5	1.3	2.0	-
16:2ω4	1.7	4.6	-	-	-
16:3ω4	0.5	14.8	-	-	-
16:3ω3	0.1	-	-	-	0.4
16:4ω1	0.3	1.5	-	-	-
18:2ω6	0.7	1.6	-	1.3	2.3
18:3ω6	0.1	-	-	-	0.2
18:3ω3	0.2	-	1.4	1.4	0.4
18:4ω3	4.0	0.6	1.1	3.8	8.0
20:2ω6	0.3	-	0.6	-	-
20:3ω6	0.1	1.7	-	0.6	-
20:3ω3	0.1	-	-	-	-
20:4ω6	0.3	-	0.8	0.5	0.1
20:4ω3	0.1	0.5	-	1.2	-
20:5ω3	16.3	18.9	9.9	12.3	7.2
22:2ω6	0.5	-	-	-	-
22:5ω6	1.2	-	1.4	1.0	-
22:5ω3	0.4	0.5	-	-	-
22:6ω3	13.1	3.3	3.9	5.7	4.3
Total	40.2	50.5	19.8	29.8	22.9
Other saturated					
15:0 Branched	-	0.5	-	-	-
17:0 Branched	-	0.3	-	-	1.1
Total of ω6	3.2	3.8	2.8	3.4	2.6
Total of ω3	34.3	23.3	16.3	24.4	20.3
ω6/ω3	0.09	0.16	0.17	0.14	0.13

compositions were qualitatively similar. *P. paradoxa* and *Chlorella* sp. showed some differences in proportion of certain components. In addition, the proportion of total polyethylenic acids in *P. paradoxa* was lower. In a comparison of the total lipid and total protein (47) of individual algal cells, *P. paradoxa* had higher levels of total lipid/cell whereas *P. virginica* had more total protein/cell. Variations in relative proportions of certain amino acid components were also observed among these 3 algal species (47). These results suggest that a diet consisting of several species of algae is required by oyster larvae to obtain an optimal balance of all chemical components during development and metamorphosis. We

TABLE VI

Comparison of Total Weight Percentage ω6 and ω3 Fatty Acid Families in Algae, *P. paradoxa*, *P. virginica*, *Chlorella* sp., *M. lutheri* and *I. galbana*

	Algal species									
	P. paradoxa		*P. virginica*		*Chlorella* sp.		*M. lutheri*			*I. galbana*	
Fatty acids	10th[a]	15th	10th	15th	10th	15th	(20)[b]	(23)	(Joseph)	(22)	(Joseph)
Total ω6	5.13	3.45	8.65	5.65	11.48	8.90	3.20	3.80	2.80	1.10	3.40
Total ω3	8.80	7.95	27.05	25.35	23.89	20.80	34.30	23.30	16.30	20.30	24.40
ω6/ω3	0.58	0.43	0.32	0.22	0.48	0.43	0.09	0.16	0.17	0.13	0.13

[a]10th, 15th days algal cultures.
[b]References 20, 21, 23 and Joseph, personal communication.

also found there are quantitative differences among certain fatty acids that were present in both the "good food" and the "mediocre food;" this result indicated a possible role of lipid in explaining the difference between the "good" and the "mediocre" algae as food for oysters. Since knowledge concerning the nutritional requirements of oysters and oyster larvae is still limited, a definite conclusion cannot be drawn until a complete analyses of the 3 chemical components (protein, lipid and carbohydrate content) of the algae, as well as the effect of vitamins and minerals on the growth of oyster larvae, have been performed. It is also necessary to perform the same kind of chemical analyses on oyster larvae maintained on different diets while they are in comparable stages of growth.

ACKNOWLEDGMENT

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The Adsorption of Bile Salts on Activated Carbon

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ABSTRACT

Activated carbon (AC) has been shown to be effective in reducing serum cholesterol and triglycerides. The mechanism for this action is proposed to be a result of the removal of bile salts in the gut. In this paper, the adsorption of cholate, glycocholate, taurocholate, chenodeoxycholate and deoxycholate on AC is studied in vitro. The results indicate that AC has a high capacity for bile salts, completely removing them from solutions of up to 5 mM and at a rate consistent with physiological activity. Of the 2 types of AC tested, one was shown to exhibit greater capacity and selectivity over the other. A negligible effect was seen with variation of pH through the range 7-9. Desorption occurs in the presence of bile salt-free buffer, but to a minimal extent. Based on these data, the adsorption of bile salts by AC appears to be a likely mechanism for AC-induced reduction of serum lipids.

INTRODUCTION

Recently, in vivo studies have demonstrated that serum levels of cholesterol and triglycerides can effectively be reduced by administration of activated carbon (AC), either alone (1) or in combination with other materials, such as oxidized starch (2,3). The results of these studies suggest that AC is chiefly responsible for the observed reduction in serum lipids.

Because this material has long been known to be safe and relatively free of side effects when administered orally (4), its potential use as a hypolipidemic agent is of considerable interest. Nevertheless, little is known about the mechanism by which this effect is accomplished. Friedman et al. (3) have suggested that AC might adsorb bile salts in the gut. This would prevent the emulsification and intestinal absorption of ingested lipids. In this study, the adsorption by AC of bile salts in solution has been investigated in vitro. The results indicate that AC is capable of adsorbing bile salts in sufficient quantity to explain the observed in vivo effect.

MATERIALS AND METHODS

Materials

Two types of AC were evaluated. Most studies were done using a wood charcoal (activated charcoal, USP, City Chemical Corporation, New York, NY). Additionally, a petroleum-based carbon, Witco Grade 517 (Witco Chemical Corporation, New York, NY), previously used in clinical studies (5) was compared with the wood charcoal in one group of experiments. The City Chemical product (CAC) was supplied as a very fine powder (>90% through U.S. standard sieve #325 mesh). The Witco carbon (WAC) was obtained in granular (8 x 30 mesh) form and pulverized to 80 x 325 mesh for these experiments. Cholestyramine was obtained from Merck & Co., West Point, PA (Cuemid, T.M., Merck, Sharpe and Dohme Division).

The bile salts studied were cholate (C), glycocholate (GC), taurocholate (TC), deoxycholate (DC) and chenodeoxycholate (CDC). These were obtained as the sodium salts (Calbiochem-Behring Corporation, La Jolla, CA) and were determined to be chromatographically pure (by thin layer chromatography, courtesy of Drs. K. Prakash and R. Lastomirsky, Division of Digestive Diseases). In order to duplicate as closely as possible physiological conditions in the gut, a bicarbonate-based buffer system was used. This buffer (BBS) was made by the addition of 50 ml of a solution containing 4.2 g sodium bicarbonate (Bristol Labs, Division of Bristol Meyers Company, Syracuse, NY) to 1 ℓ of 0.9% w/w aqueous NaCl (NS). BBS, stored in stoppered bottles, was shown to exhibit daily a pH of 7.98 - 8.02 over the course of 1 wk. This buffer was used with C, GC, TC and CDC but the pH was increased to 8.34 ± 0.04 (with 0.1 N NaOH) for DC because of its limited solubility at lower pH. For experiments at pH 9, the BBS was adjusted with 0.1 N NaOH. Bile salt solutions of pH 7 (for 10 mM) were obtained by dissolving the bile salts in NS.

Methods

Incubations were carried out in a static system similar to that described by Birkner and Kern (6). Rubber-stoppered borosilicate glass

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test tubes containing AC and bile salt solution (5 ml) were incubated at 37 C with moderate agitation in a Dubnoff metabolic incubator. The AC was added to the tube, followed by the bile salt solution and the tubes were shaken with a vortex mixer to affect suspension of the AC prior to incubation. At the completion of the incubation period, the suspensions were gravity filtered through 2 layers of Whatman #541 filter paper and refiltered when necessary.

The amount adsorbed was calculated from the final concentration of bile salt solution which was measured by forming the sulfuric acid chromogens in concentrated H_2SO_4 (7). These were then assayed spectrophotometrically at 388 nm. It was found that linear standard curves were obtained for bile salt solutions up to 10 mM using reaction mixtures of 10 μl trihydroxy bile salts (C, GC and TC) + 3 ml H_2SO_4 (95-98%, AR grade Mallinckrodt Chemical Corporation, St. Louis, MO) and 100 μl dihydroxy bile salts (DC and CDC) + 3 ml H_2SO_4. For studies at higher concentrations, dilutions were made. All measurements were made with a model 25 double-beam spectrophotometer (Beckman Instruments, Fullerton, CA) vs an appropriate reagent blank.

RESULTS

In the first group of experiments, the rate of adsorption was determined at constant (10 mM) bile salt concentration, and for 2 different AC loadings, 0.05 and 0.5 g. These studies were done not only to determine the length of time AC would take to reach equilibrium adsorption with bile salts in the intestinal tract, but also to ascertain what equilibration time should be used for the remainder of the static incubations in this study. The results are in Figure 1. For both loading groups, equilibrium is rapidly approached during the first 30 min of incubation and is achieved within the first hr. Based on these experiments, it was decided that a 2-hr incubation time would guarantee adsorption equilibrium. Also it can be seen from Figure 1 that 0.5 g of AC completely removes bile salts from solution, but this quantity is probably too high a loading dosage. Birkner and Kern (6), using a similar static incubation system, employed a loading of 0.1 g for cholestyramine and it was decided that this dosage would be not only more realistic in terms of oral administration, but would also allow comparison with the data on cholestyramine adsorption.

The capacity of CAC to adsorb bile salts was studied at concentrations up to 10 mM for CDC and DC and up to 35 mM for C, GC and TC. For C, GC and TC solutions of 1 mM and below and for CDC and DC solutions of 2 mM and below, the final (equilibrium) concentrations were zero. This largely prohibits the application of the reciprocal Langmuir isotherm (8,9) as was previously done (6), since a large number of indeterminate points would result. The results were therefore plotted as specific adsorption (wt of adsorbate/wt of adsorbent) vs initial concentration. These are shown in Figure 2. The data demonstrate that, in solutions of up to 5 mM, virtually all the bile salt is removed by the AC, resulting in the linear portion of the curve. At higher concentrations, a saturation effect is seen, i.e., the curves begin to level off. Theoretically, the maximal amount of bile salt capable of being adsorbed can be computed from a double reciprocal plot of the coordinates used in Figure 2. These data are given in Table I for C, GC and TC. The maximal capacities of DC and CDC are not given, since the 10 mM points are too far from the plateau portion of the curve. In fact, when this analysis was performed on the dihydroxy bile salts, values of

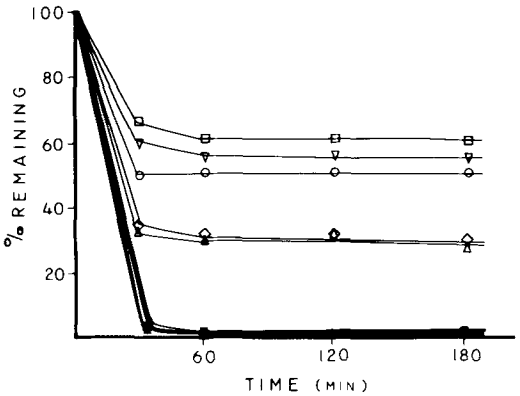

FIG. 1. Rate of adsorption of bile salts on CAC. Open symbols are C (○), GC (□), TC (▽), CDC (◇) and DC (△) at 10 mM on 0.05 g AC. Solid symbols are for the same bile salts on 0.5 g AC.

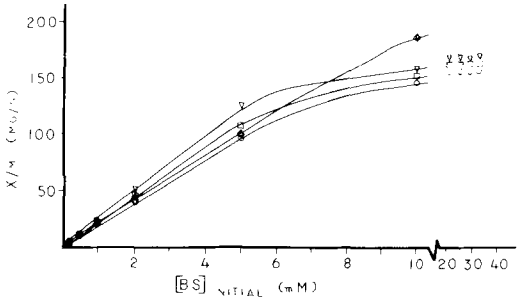

FIG. 2. Isotherms of specific adsorption (X/m) vs initial concentration. The symbols used are the same as in Figure 1.

TABLE I

Adsorption Capacity of Bile Salts on CAC at 10 mM Bile Salt Concentration and at the Theoretical Maximum

Bile salts	Capacity, X/m (mg BS/g AC) @ 10 mM[a]	Maximum[b]
C	145 ± 6.1	366
GC	151 ± 8.4	408
TC	156 ± 8.5	417
CDC	179 ± 5.9	-
DC	181 ± 6.3	-

Significance levels for bile salt pairs @ 10 mM[c]

C vs GC; NS[d]	GC vs TC; NS
C vs TC; NS	GC vs CDC; $P < 0.01$
C vs CDC; $P < 0.001$	GC vs DC; $P < 0.01$
C vs DC; $P < 0.001$	TC vs CDC; $P < 0.01$
CDC vs DC; NS	TC vs DC; NS

[a]Mean ± 1 SD, n = 5.

[b]From regression of c^{-1} vs m/X; @ $c^{-1} = 0$.

[c]By t-test.

[d]NS (not significant); $P \geqslant 0.05$.

TABLE II

Adsorption of Bile Salts on Cholestyramine and Comparison with CAC

Bile salts	@ 5 mM X/m[a]	@ 5 mM RMA[b]	@ 10 mM X/m	@ 10 mM RMA
C	98	1.03	193	1.33
GC	114	1.07	222	1.47
TC	128	1.02	248	1.59
CDC	98	1.00	190	1.06

[a]Capacity in mg BS/g AC.

[b]Relative Molar Adsorption = moles adsorbed on cholestyramine/moles adsorbed on CAC, per unit wt of adsorbent.

over 0.5 g/g AC were obtained. It should be stressed that these data represent theoretical values for a system in which a high enough bile salt concentration is used to obtain the maxima. It is unlikely that these values would be reached under physiological conditions.

Table I also gives the results of a series of bile salt (10 mM) incubations on CAC. This was done to determine whether there were any statistically significant differences among the bile salts using CAC as an adsorbent. As can be seen from the data, the dihydroxy bile salts are adsorbed to a much greater extent than the trihydroxy bile salts, whereas there was no significant difference among C, GC and TC and again no significant difference between DC and CDC.

A comparative study of bile salt adsorption on cholestyramine was carried out. At initial concentrations up to 5 mM, this resin completely removes bile salts from solution, as does CAC. At 5 mM and 10 mM, however, cholestyramine adsorbs greater amounts of bile salt than does CAC. These data are presented in Table II. At 5 mM, slightly more of the trihydroxy salts (C, GC and TC) are bound to the resin than are adsorbed to CAC; there is no difference with the dihydroxy salt studied (CDC). At 10 mM, cholestyramine adsorbs much more of the C, GC and TC than does CAC, although only slightly more CDC is adsorbed.

The ability of CAC to adsorb bile salts was compared with WAC for 10 mM solutions of C and CDC. The results, shown as the equilibrium bile salt concentrations, are given in Figure 3.

These data demonstrate large differences between the abilities of these products to adsorb bile salts ($P < 0.001$); the CAC is capable of adsorbing far more bile salt/wt of adsorbent.

Experiments were also run at pH 7 and pH 9 to determine whether the pH, in range 7-9, would affect the adsorption of C, GC, TC and CDC to CAC. These data are shown in Figure 4. Although a general trend showing that, at pH 8, maximal adsorption takes place, i.e., the equilibrium concentration is lowest (with the exception of C) at pH 8, the data for all 3 pH points is within 2 SD of the pH 8 data, indicating the difference is probably not significant.

Desorption experiments were run at 40 C on 0.5 g samples of CAC which had been allowed to equilibrate with 10 mM (5 ml) solutions of C and CDC. These samples were then placed in an apparatus allowing a constant influx as well as efflux of BBS. Prior to the initiation of BBS flow (20 ml delivered from a metering pump over the course of 1 hr), the compartment containing the CAC was filled with 5 ml of BBS and allowed to incubate for 15 min. Thus, the conditions allowing analysis by a first-order mathematical model (10) were maintained. Ten fractions of 2 ml each were collected. These were assayed and the data obtained are shown in Figure 5. These data demonstrate an initial leaching of bile salt during the 15 min incubation with little or no desorption thereafter. The first-order model was applied and, based on gravimetrically measured void volume (pores + interstitial space) of 1.54 cc/g, the data shown in Table III were calculated. These indicate that the amount desorbed from both C and CDC are less than 0.01% of the original amount adsorbed.

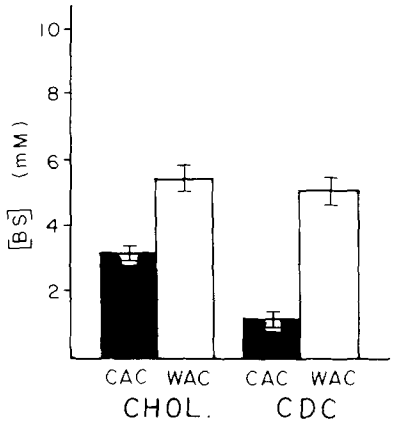

FIG. 3. Equilibrium concentrations of bile salt solutions after adsorption on CAC and WAC for C and CDC ($P < 0.001$ for both AC pairs).

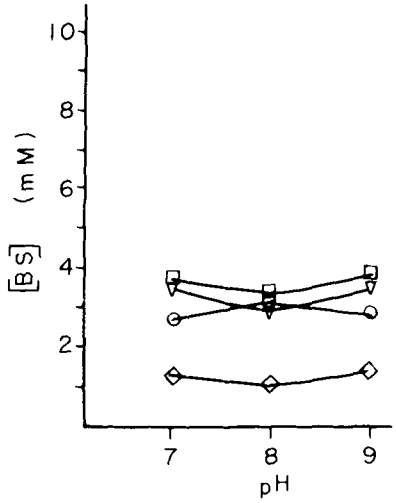

FIG. 4. The effect of pH on equilibrium bile salt concentration after adsorption on CAC.

DISCUSSION

The importance of substances which bind bile salts in the gut is well established. A considerable amount of work has been done in the area of food residue (fiber) binding of bile salts, both in vivo (11-14) and in vitro (6,15-17), and although these substances reliably increase fecal excretion of bile salts, reduction of serum lipids is not always observed (18). The finding that AC reduces cholesterol and triglycerides in serum (1-3) is important in that AC given orally has a history of safe use.

The mechanism of hypolipidemic action of AC has hitherto been unexplored. Direct binding of foodstuff lipids is an extremely unlikely event. AC is a microporous carbon with a microcrystalline matrix derived from the basic graphite structure (19). Because of its

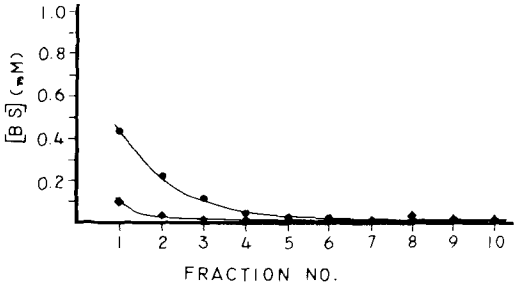

FIG. 5. Desorption of C (•) and CDC (♦) from CAC.

TABLE III

Desorption of C and CDC from CAC

Bile salt	[BS]i[a] (mM)	BS desorbed (mg)	% desorbed[b]
C	0.67	1.73×10^{-3}	0.0085
CDC	0.22	0.49×10^{-3}	0.0025

[a]BS concentration after 15 min incubation with 5 ml BBS.

[b]BS desorbed/BS adsorbed x 100, based on total wt of CAC (0.5 g).

extensive microporosity (pores of 500 Å or less), it has surface areas which may approach 2000 m^2/g for some carbons. Most of this area, however, is in pores which would be inaccessible to particles of the size which would occur in emulsions. A much more plausible explanation is that soluble substances, directly or indirectly responsible for the adsorption of lipids by the gut luminal epithelium, are being removed. Two groups of substances which are likely candidates for this role are the soluble lipids themselves, such as fatty acids, and the emulsifying agents, i.e., the bile salts. Soluble lipids have been extensively studied (20) in the past. In fact, adsorption of both dietary free fatty acids or fatty acids from lipase-degraded dietary triglycerides may contribute to the reduction of these in serum but should have little or no effect on serum cholesterol. This leaves bile salt adsorption as the most likely mechanism.

The results presented in this work show that bile salts are rapidly and effectively adsorbed to AC. Comparison of this data (Fig. 2) with that for cholestyramine (Table III) indicates that AC binds bile salts to the same degree at initial concentrations up to 5 mM and, in general, has a high capacity for bile salts.

The type of AC used has been shown to have a significant effect. CAC and WAC, on a weight basis, do not adsorb bile salts to the same degree. WAC, however, has a much greater apparent density and might be nearly as effective on a volume basis. These aspects, i.e., comparative studies of different types of AC, require further study. It would also be worthwhile to study whether there are differences in the affinities of the various carbon surfaces for different bile salts. CAC shows a much higher capacity for dihydroxy than trihydroxy bile salts whereas WAC adsorbs both C and CDC to nearly the same degree.

Desorption experiments were carried out using somewhat more stringent conditions than those used on lettuce residue (6); bile salt-free buffer was used to desorb instead of diluted bile salt solutions. This may account for the finding that some bile salts were desorbed. The amount removed, howver, would probably be neither observed in vivo (because access of intestinal secretions to the adsorbed bile salts would be limited by the dispersion of the AC in the fecal mass) nor significant, in that quantitatively it represents a small fraction of the initial bile salt present.

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Effect of Oxidized Oil on Lipogenic Enzymes

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ABSTRACT

Male Wistar rats were fed for 4 wk on diets containing 2% oxidized corn oil. Liver tissue was then studied to determine the effect of feeding peroxidized oil on lipogenic enzymes. Although substances which reacted with thiobarbituric acid increased in liver microsomes and mitochondria with increasing peroxide values of the dietary corn oil fed, the activities of glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase in liver were unchanged. However, when rats were fed for 2 wk on diets containing 10% fat, of which 0.5, 5 or 10% was unoxidized corn oil and the remainder was hydrogenated beef tallow filler, the lipogenic enzyme activities and also the liver triglyceride levels were observed to decrease with increasing amounts of dietary corn oil. Therefore, although a synthetic diet containing corn oil was easy to oxidize spontaneously, the reductions of lipogenic enzymes in rats fed the diet would not have been caused by lipid peroxides but by unsaturated fatty acids themselves.

INTRODUCTION

The activities of rat liver lipogenic enzymes such as acetyl-CoA carboxylase, malic enzyme and glucose-6-phosphate dehydrogenase have been observed to decrease as a result of feeding polyunsaturated fat (1). It was suggested that fatty acids act in the short-term inhibition as well as in the adaptive inhibition of fatty acid synthesis (2). Polyunsaturated fatty acids caused less inhibition than did saturated fatty acids in the short-term inhibition of fatty acid synthesis in liver (3-5). In the long-term regulation, however, the lipogenic enzymes were lowered to a greater extent by feeding polyunsaturated fatty acid (1,6,7). C_{20}-Unsaturated fatty acids were more inhibitory in short-term regulation of fatty acid synthesis than C_{16}- or C_{18}-saturated fatty acids (8).

Synthetic diets containing fats rich in polyunsaturated fatty acids such as corn oil peroxidize easily, as shown in the results of this study. The results suggest that animals fed such experimental diets may be consuming lipid peroxides which form spontaneously to a greater or lesser extent, depending on the conditions of diet storage. The possibility that feeding diets containing peroxidized fat may be an important factor in the influence of unsaturated fat on lipogenic enzymes was considered. In this study, we have tried to determine whether or not dietary lipid peroxides have a significant effect in the reduction of lipogenic enzyme activities of rats fed diets containing corn oil.

MATERIALS AND METHODS

The diets containing 0, 5 or 10% corn oil were put in feeding dishes (7 cm id and 4.5 cm deep) and placed at either 4 or 25 C. Peroxide values of the diets were determined by iodometry (9) at days 0, 1, 3, 6, 10, 20 and 30. The diets contained 68.4% sucrose, 18% casein, 9.5% cellulose, varying amounts of corn oil at the expense of sucrose, 4% salt mixture, 0.1% choline chloride and vitamin mixture. The salt mixture contained: (%) $CaCO_3$, 29.29; $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $MgSO_4 \cdot 7H_2O$, 9.98; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.623; $CuSO_4$, 0.156; $MnSO_4 \cdot H_2O$, 0.121; $ZnCl_2$, 0.020; KI, 0.0005; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0025; $Na_2SeO_3 \cdot 5H_2O$, 0.0015. Vitamins added per 100 g diet of ration: retinyl palmitate, 2,000 IU; (mg) calciferol, 0.01; D,L-α-tocopherol 8; thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 8.0; folic acid, 0.25; calcium pantothenate, 2; biotin, 0.5; inositol, 4.0; menadione, 0.25.

In experiment 1, 5-wk-old Wistar rats were fed for 4 wk on 2% corn oil diets, containing varying amounts of peroxides. Corn oil was oxidized by exposure to ultraviolet irradiation with constant shaking at 37 C for 2-6 days prior to addition into the diet. In experiment II, rats were fed for 2 wk on 1 of 3 diets which contained, respectively, 0.5, 5 or 10% corn oil (peroxide-free) and hydrogenated beef tallow so that the total fat content of all diets was 10%. The diet compositions in experiments I and II were the same as already mentioned. Rats in groups A, B and C were pair-fed and kept under standardized laboratory conditions (24 C, automatic lighting schedule from 7 a.m. to 7 p.m.). Rats were killed by decapitation between 10 a.m. and 11 a.m. The liver was quickly removed for the assay of lipogenic enzyme activities in the 105,000 x g supernatant fraction.

Glucose-6-phosphate dehydrogenase (EC

1.1.1.49) was assayed according to Glock and McLean (10), and malic enzyme (EC 1.1.1.40) according to Ochoa (11). Acetyl-CoA carboxylase (EC 6.4.1.2) was assayed at 37 C by the $H^{14}CO_3^-$-fixation method (12). Total lipids of liver were extracted as described by Folch et al. (13). Liver and serum triglycerides were determined according to Fletcher (14). Thiobarbituric acid (TBA)-reacting compounds were measured according to Takeuchi et al. (15) and Ohkawa et al. (16).

RESULTS AND DISCUSSION

The time courses of peroxide values of diets containing corn oil are shown in Figure 1. The 0, 5 or 10% corn oil diets autoxidized quickly at 25 C and slowly at 4 C when the diets contained 80 mg D,L-α-tocopherol/kg diet. The peroxide formation in the 10% corn oil diet was significant even after 1 day at 25 C. A possible factor in this effect may be that the surface of the corn oil was greatly increased by the adsorption of granules of sugar and casein, as well as provided with abundant oxygen. Therefore, conditions under which the diet is kept are important in order to minimize peroxide formation.

The levels of TBA-reacting compounds in serum and liver were assumed to represent the peroxide levels in this study. In experiment I, when the rats were fed on 2% fat diets containing varying amounts of peroxides (TBA values and iodometry peroxide values), TBA-reacting compounds in liver microsomes and mitochondria were increased with increasing dietary peroxides, as shown in Table I. Dietary TBA-reacting compounds could be absorbed and accumulated. We also demonstrated in a previous experiment that when rats with lymph fistulae were given orally oxidized oil, TBA-reacting compounds were recovered in thoracic lymph (unoxidized oil, TBA-reacting compounds were recovered in thoracic lymph

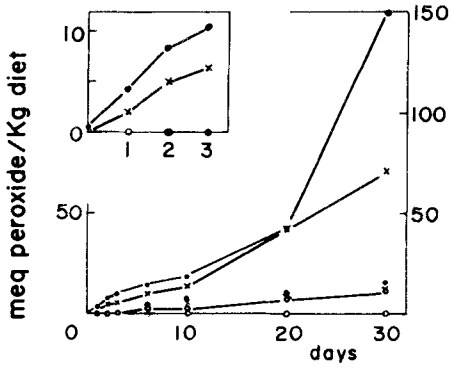

FIG. 1. Time courses of peroxide values in diets containing varying amounts of corn oil. The diets containing 0% (○), 5% (x) or 10% (●) corn oil were placed in diet containers (7 cm id and 4.5 cm deep) at 4 C and at 25 C. Peroxide values at 25 C are plotted with lines connecting the different symbols. Line connecting the symbols for diets stored at 4 C are omitted. Peroxide values at days 1, 2 and 3 are separately shown in the enlargement to upper left.

TABLE I

Effect of Dietary Peroxides and Corn Oil on TBA-Reacting Compounds in Rat

Dietary TBA-reacting compounds	TBA-reacting compounds		
	Serum	Liver	
		Microsomes	Mitochondria
	μmol/100 g diet	nmol/ml	nmol/mg protein
(I) A 0	2.61 ± 0.11	0.68 ± 0.08	0.27 ± 0.06
B 0.05 (421)	2.81 ± 0.28	1.66 ± 0.60[b]	0.55 ± 0.25[a]
C 0.10 (842)	2.91 ± 0.45	3.61 ± 0.49[c,d]	0.61 ± 0.05[c]
Dietary (%)			
(II) A 0.5	2.10 ± 0.17	0.39 ± 0.06	0.26 ± 0.08
B 5	2.48 ± 0.16[b]	0.73 ± 0.12[c]	0.36 ± 0.07[a]
C 10	3.33 ± 0.22[c,d]	1.79 ± 0.38[c,d]	0.43 ± 0.04[c]

In experiment I, rats were fed for 4 wk on 2% corn oil diets containing varying amounts of TBA-reacting compounds.

In experiment II, rats were fed for 2 wk on 10% fat diets containing 0.5, 5 or 10% corn oil (hydrogenated beef tallow made up the balance of the fat in the diet).

Peroxide values measured iodometrically are shown in parenthesis.

Values differing significantly from A: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

Values differing significantly from B: [d]$P < 0.001$.

Mean ± SD for 6 rats.

TABLE II

Effect of Dietary Peroxides and Corn Oil on Lipogenic Enzymes

	Dietary TBA-reacting compounds	Glucose-6-phosphate dehydrogenase[a]	Malic enzyme[a]	Acetyl-CoA carboxylase[b]
	μmol/100 g	nmol/min/mg at 37 C		
(I)	A 0	330 ± 69.0	318 ± 65.6	19.4 ± 4.35
	B 0.05 (421)[c]	318 ± 52.3	299 ± 63.0	16.9 ± 2.69
	C 0.10 (842)	346 ± 23.9	287 ± 11.9	20.2 ± 2.05[f]
	Dietary corn oil (%)			
(II)	A 0.5	386 ± 26.4	282 ± 25.5	18.5 ± 1.50
	B 5	141 ± 20.5[e]	148 ± 21.5[e]	16.0 ± 1.01[d]
	C 10	76.6 ± 1.80[e,h]	91.7 ± 12.0[e,h]	12.5 ± 2.30[e,g]

See Legend in Table I.
Values differing significantly from A: [d]$P < 0.01$; [e]$P < 0.001$.
Values differing significantly from B: [f]$P < 0.05$; [g]$P < 0.01$; [h]$P < 0.001$.
[a]nmol substrate utilized.
[b]nmol product formed (enzyme activities in 105,000 x supernatant fraction of liver homogenate).
[c]Peroxide values measured iodometrically are shown in parentheses.
Mean ± SD for 6 rats.

(unpublished). The activities of glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase in the rat liver were not decreased by increasing dietary peroxides, as shown in Table II. In experiment II, when rats were fed on 10% fat diets containing varying amounts of corn oil, TBA-reacting compounds in liver microsomes and mitochondria as well as in serum were increased with increasing amounts of dietary corn oil. With the increased dietary corn oil, the activities of glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase were reduced significantly, as shown in Table II. Serum and liver triglyceride levels of the rats were also decreased by increasing dietary corn oil, as shown in Table III. These results suggest, therefore, that lipogenic enzyme activities and triglyceride levels have no relationship to lipid peroxides but were reduced by corn oil unsaturated fatty acids per se.

Chow et al. (17) reported that when rats were fed on a 15.7% corn oil diet with α-tocopherol, the activities of glucose-6-phosphate dehydrogenase were significantly increased in adipose tissue and muscle, but were not changed in liver compared to α-tocopherol-fed controls. In their experiments, the enzyme activities were well correlated with glutathione peroxidase activity, which has been related to lipid peroxidation (15,18-20). Since the membranes of subcellular organelles contain unsaturated phospholipids, peroxidation of phospholipids might induce membrane damage and impairment of related enzymes. Glucose-6-phosphate has been shown to be sensitive to membrane alteration and was decreased with increasing peroxides (15,21). However, it is suggested in the work of Chow et al. (17) that, although liver peroxide levels should be elevated in animals fed the corn oil diet without α-tocopherol, liver glucose-6-phosphate dehydrogenase was not decreased. The experiments in this report indicate that liver glucose-6-phosphate dehydrogenase is not altered by TBA-reacting compounds. Therefore, we can discount the possibility that lipid peroxidation and lipoperoxides decreased the lipogenic enzymes in liver. It is suggested that lipogenic enzyme activities were reduced by the un-

TABLE III

Effect of Dietary Peroxides and Corn Oil on Triglyceride Levels in Rats

	Dietary TBA-reacting compounds	Triglycerides	
		Serum	Liver
	μmol/100 g diet	mg/ml	mg/g
(I)	A 0	1.45 ± 0.45	16.9 ± 5.32
	B 0.05 (421)[b]	1.51 ± 0.26	17.6 ± 6.55
	C 0.10 (842)	1.62 ± 0.61	18.0 ± 6.64
	Dietary corn oil (%)		
(II)	A 0.5	2.33 ± 0.57	19.9 ± 3.90
	B 5	2.51 ± 0.11	10.1 ± 0.61[a]
	C 10	2.50 ± 0.22	9.53 ± 0.19[a]

See Legend in Table I.
Values differing significantly from A: [a]$P < 0.001$.
[b]Peroxide values measured iodometrically are shown in parentheses.
Mean ± SD for 6 rats.

saturated fatty acids themselves.

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Sterol Composition of Ungerminated and Germinated Spores of the Vesicular-Arbuscular Mycorrhizal Fungus, *Glomus caledonius*

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ABSTRACT

The sterol composition of spores of the Vesicular-Arbuscular Mycorrhiza, *Glomus caledonius*, have been examined by combined gas liquid chromatography-mass spectrometry (GLC-MS). The sterols identified were cholesterol, 24-methylcholesterol, 24-methylenecholesterol and 24-ethylcholesterol. The sterols 24-methylcholesterol, 24-methylenecholesterol and 24-ethylcholesterol have not previously been reported as components of fungi in the Zygomycotina. The spores were germinated on agar and the major changes in sterol esters, free and bound sterols were studied over a period of 21 days. The total sterol content continually increased during the growth period; 24-methylcholesterol and cholesterol were shown to be the major sterols.

INTRODUCTION

In recent years, there has been a rapidly growing interest in the biology of Vesicular-Arbuscular (VA) endophytic fungi, which form mycorrhizas within plant roots. These fungi facilitate the uptake of nutrients, particularly phosphorus, by the plant (1).

There are several reports on the lipids in these fungi (2,3); however, there are no reports on their sterol composition. McCorkindale et al. examined a number of other fungi of the order Mucorales and showed cholesterol, ergosterol and 22-dihydroergosterol to be the major sterols (4). Species of Mucorales such as *Rhizopus arrhizus* contained fungisterol, 5-dihydroergosterol, ergoesterol and ergosta-$\Delta^{5,7,14(15)}$-trienol (5) and *Phycomyces blakesleeanus* contained episterol (6) and ergosta-$\Delta^{5,7,24(28)}$-trienol (7). Fungisterol was the major sterol in the conidia of *Linderina pennispora*, but no ergosterol was detected (8).

This paper describes the sterol esters, free and bound sterols of *Glomus caledonius* spores during germination and germ tube growth.

EXPERIMENTAL PROCEDURES

Spore Production and Isolation

Glomus caledonius spores were grown on the roots of *Trifolium subterraneum* (9) in soil which had been steam treated. The spores were isolated from air-dried soil by centrifugation in 50% (w/v) sucrose solution and sterilized as previously described (9).

Culture Conditions

Soil extract agar media was prepared as previously described (10), except that it contained chloramphenicol at 100 μg/ml. Surface-sterilized spores were placed on type HA Millipore filter segments (Millipore Corporation, Bedford, MA) overlying the agar which enabled easy harvesting of the spores (9). The inoculated petri dishes were enclosed in plastic bags and incubated in the dark at 20 ± 2 C.

Spore Harvest

G. caledonius spores were examined microscopically at a magnification of 200X and removed from the Millipore filters with a fine sable hair brush. Harvest times were after 7, 14 and 21 days of incubation. Spores were dried in vacuo over KOH pellets for 24 hr and then weighed. Spore numbers used in these experiments were 4,000 ungerminated spores, 2,000 day-7 spores, 1,800 day-14 spores and 1,000 day-21 spores.

Sterol Extraction

Immediately after harvesting, the spores were disrupted in a glass screw-capped mortar and pestle. The contents were checked microscopically to ensure that all spores were fractured, and then extracted using the Renaud et al. solvent system (11). The lipid extract was chromatographed on glass plates coated with Silica Gel G (containing a fluorescent indicator) and developed with hexane/diethyl ether/methanol (40:10:1). Free sterols were recovered from the silica gel in diethyl ether. The solvent was evaporated, the extract dissolved in 1 ml of chloroform and rechromatographed on Silica Gel H with hexane/diethyl ether/acetic acid (35:15:0.5) containing the fluorescent dye 2,5-bis[5′-*tert*-butyl-benz-oxazolyl-(2′)]-thiophene (BBOT). Sterols were recovered from the second chromatogram as before.

Sterol esters from the chromatogram were hydrolyzed in 10% KOH in 95% ethanol (2 ml) at 90 C for 2 hr. Free sterols were recovered in light petroleum (bp 30-60 C), dried, evaporated and dissolved in chloroform. This fraction was then rechromatographed as before. Bound sterols were recovered from the solvent-extracted spore residue by hydrolysis in 10% KOH in 95% ethanol (5 ml) at 90 C for 2 hr. Free sterols were recovered and chromatographed as before.

The 3 sterol fractions were acetylated with pyridine/acetic anhydride (2:1 v/v) overnight. Sterols were identified using gas liquid chromatography (GLC) for comparison of relative retention times with authentic standards and/or cholesteryl acetate (12). The amount of individual sterols was determined by comparison of peak areas to that of stigmasterol, the internal standard. The identity of the major sterol components was confirmed by mass spectroscopy (MS) on a Varian Matt 311 mass spectrometer, using an ionization voltage of 70 eV.

RESULTS AND DISCUSSION

Sterol Identification

Sterols were extracted from spores of *Glomus caledonius* and isolated by thin layer chromatography (TLC). Preparative TLC on Silica Gel H gave one band with an R_f of 0.17. The sterols from this band were acetylated and separated by GLC using a 3% SE-30 column (1.8 m x 3.4 mm). Tentative identifications based on retention data (Table I) of the 4 sterols resolved were cholesterol, 24-methylenecholesterol, 24-methylcholesterol and 24-ethylcholesterol (12). The mass spectrum of the first sterol acetate eluted on the SE-30 column showed ion peaks at m/e 368 [M^+-(acetate)], 353 [M^+-(CH_3 + acetate)], 255 [M^+-(side chain + acetate)], and 247 [M^+-(acetate + C_9H_{13})], confirming this sterol as the acetate derivative of cholesterol.

The second sterol acetate eluted showed a mass spectrum 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 diunsaturated C_{28} sterol acetate with one double bond in the steroid nucleus. Also, an ion peak at m/e 296 [M^+-(84 + acetate)] was caused by loss of part of the side chain which resulted from MacClafferty rearrangement (13). Brassicasterol acetate and 24-methylenecholesterol acetate have almost identical mass spectra (14) but can be distinguished by their GLC relative retention data since 24-methylenecholesterol acetate has a longer retention time than brassicasterol (12). Therefore, the structure was assigned as 24-methylenecholesterol.

The major sterol component of the ungerminated spores (Fig. 1) gave a GLC relative retention time that corresponded to 24-methylcholesterol. The mass spectrum of the sterol acetate gave ion peaks at m/e 382 [(M^+-(acetate)], 367 [M^+-(CH_3 + acetate)], 255 [M^+-(side chain + acetate)], and 213 [M^+-(side chain + 42 + acetate)], indicating a monounsaturated C_{28} sterol acetate with the double bond in the steroid nucleus, thereby confirming the structure to be 24-methylcholesterol.

It was not possible to assign a definite structure to the fourth sterol component because the compound was present in small amounts. However, using GLC relative retention time data, it was provisionally identified as 24-ethylcholesterol.

In the ungerminated spores, 24-methylcholesterol was the major free sterol (95%) with lesser amounts of cholesterol (2.6%), 24-methylenecholesterol (1.8%) and 24-ethylcholesterol (0.4%). In both the sterol ester and bound sterol fractions, cholesterol was the major sterol (63% and 80%, respectively), followed by 24-methylcholesterol (37% and

TABLE I

Relative Retention Times of Sterol Acetates[a]

Sterol acetate	3% SE-30 column[b]	Literature values[c]
Cholesterol	1.00	1.00
24-Methylenecholesterol	1.26	1.26
24-Methylcholesterol	1.29	1.30
Stigmasterol[d]	1.41	1.42
24-Ethylcholesterol	1.62	1.63

[a]Relative to cholesteryl acetate (ret. time = 54.9 min).

[b]On a column of 3% SE-30 on Gas Chrom Q (1.8 m x 3.4 mm) (12).

[c]Ref. 12.

[d]Internal standard.

20%, respectively). No 24-methylenecholesterol or 24-ethylcholesterol was detected.

Sterol Changes during Germination and Germ Tube Growth

At each phase of spore germination, changes in the major sterol components (24-methylcholesterol and cholesterol) were examined, but the minor sterols 24-methylenecholesterol and 24-ethylcholesterol were not studied in detail.

Total Sterols

The total sterol content of *G. caledonius* spores steadily increased from 0.39% dry weight of ungerminated spores to 1.36% of germinated spores and germ tube dry weight after 21 days on agar (Fig. 1).

The average sterol content of fungi ranges from 0.70 to 1.0% of the tissue dry weight (15). However, McCorkindale et al. (4) showed the sterol content of several members of the Mucorales to range from zero to 0.025% of mycelium dry weight. The total sterol content of *G. caledonius* is higher than that of other Mucorales examined, but is within the general range for fungal tissue (15).

The concentration of 24-methylcholesterol was approximately double that of cholesterol in the ungerminated spores. Both sterols had increased at day 7 on agar when the germ tube first appeared. Thereafter, the concentration of 24-methylcholesterol plateaued, whereas cholesterol continued increasing, and at day 21 cholesterol was in greater concentration. Germ tube elongation ceased between 14 and 21 days, although there was an increase in total weight during this time (3).

Free Sterols

In the free sterol fraction of the ungerminated spores, the concentration of 24-methylcholesterol was ca. 24 times that of cholesterol (Fig. 1). The 24-methylcholesterol content increased up to day 7, whereas the cholesterol content increased up to day 14. The free sterol content reached a maximum of 84% of the total sterols by day 7 and a minimum of 20% by day 21.

Sterol Esters

In the sterol ester fraction, cholesterol was approximately double the content of 24-methylcholesterol (Fig. 1). The cholesterol content peaked on day 14, whereas the 24-methylcholesterol content continued increasing after day 7.

Bound Sterols

The concentration of cholesterol was 3 times that of 24-methylcholesterol in the bound sterols of ungerminated spores (Fig. 1). The levels of both sterols remained constant until day 14. Between days 14 to 21, the 24-methylcholesterol content increased 6-fold whereas the cholesterol content increased 8-fold. This large increase in bound sterols may be associated with senescence of the spores and germ tubes (16).

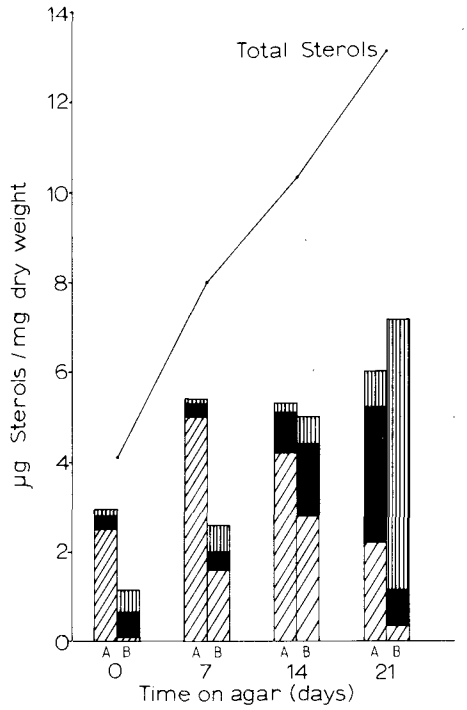

FIG. 1. Changes in total sterols, 24-methylcholesterol (A) and cholesterol (B), in free sterols (▨), sterol esters (■) and bound sterols (▥) in spores of *Glomus caledonius* during germination and germ tube growth on agar.

Previous examination of sterols of fungi in the order Mucorales has shown ergosterol to be present more frequently than cholesterol (4). However, in *G. caledonius* spores, no ergosterol was detected. Previous reports have not described 24-methylcholesterol, 24-methylenecholesterol or 24-ethylcholesterol as components of fungi in the Zygomycotina. All of these sterols have been reported in the Mastigomycotina (15).

ACKNOWLEDGMENTS

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Analysis of Oleate, Linoleate and Linolenate Hydroperoxides in Oxidized Ester Mixtures[1]

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ABSTRACT

The hydroperoxides in oxidized mixtures of methyl oleate, linoleate and linolenate were analyzed by reducing the hydroperoxides to the corresponding hydroxyesters and separating the hydroxyesters from the unoxidized esters by thin layer chromatography (TLC). The hydroxyesters from linolenate were separated from the other hydroxyesters by TLC on silver ion plates. The hydroxyesters were converted to TMS-hydroxy derivatives. The TMS-hydroxyoleate and TMS-hydroxylinoleate were separated by gas chromatography (GC), and all the TMS-derivatives were quantified by GC. The relative rates of oxidation of methyl oleate, linoleate and linolenate in mixtures were ca. 1:10.3:21.6. The hydroperoxides formed in the oxidation of soybean and olive oils were similar before and after randomization and similar to corresponding methyl ester mixtures.

INTRODUCTION

Comparisons of the rates of oxidation of purified esters of oleate, linoleate and linolenate have shown that these esters oxidize at rates in the ratios of 1:12:25, respectively (1,2). Because the rate of the initiation reaction in the oxidation of these esters was unknown, it is not certain what the relative rates of oxidation of these esters in mixtures would be. Wong and Hammond (3) measured the rate of oxidation of methyl oleate and linoleate in mixtures by reducing the hydroperoxides to the corresponding alcohols and separating the methyl hydroxyoleate from the methyl hydroxylinoleate by gas chromatography (GLC) of the trimethylsilyl (TMS)-derivatives. They found that the relative rates of oxidation of methyl oleate and linoleate seemed to vary with the composition of the mixture. Frankel et al. (4-7) also analyzed hydroperoxide mixtures by combined gas chromatography-mass spectrometry of the TMS-derivatives obtained from oxidized methyl esters after reduction of the hydroperoxide and saturation of the double bonds. They report that for methyl oleate the 4 expected isomers, 8, 9, 10 and 11-hydroxyoctadecanoate, are not formed in equal amounts, nor for methyl linolenate are the 4 expected isomers, 9, 12, 13 and 16-hydroxyoctadecanoate, formed in equal amounts. But the 2 isomers expected from linoleate, 9- and 13-hydroxyoctadecanoate, were produced in equal amouts. Further, they report that during the oxidation of soybean oil methyl esters, unexpectedly large amounts of the 12-hydroxyoctadecanoate are produced during the early stages of oxidation. Their data show that in the oxidation of mixtures of methyl oleate and methyl linoleate, the relative amounts of oleate and linoleate hydroperoxides are not proportional to the oleate and linoleate in the mixture. This suggests that the relative rates of oxidation of oleate and linoleate vary with the composition of the mixture.

METHODS

Methyl oleate, linoleate and linolenate were prepared by the urea fractionation of methyl esters from olive, safflower and linseed oils, respectively. Additional pure methyl linolenate was obtained from Nu-Chek-Prep, Inc. (Elysian, MN). Esters were purified according to Wong and Hammond (3) and oxidized in 5-g batches at 28 C unless otherwise specified. Peroxide values, iodometric reduction of the peroxides, separation of the methyl hydroxyesters, formation of the TMS-derivative, GLC and other analyses were performed as before (3).

The methyl hydroxyester from the oxidation of methyl linolenate was separated from those produced by oleate and linoleate by thin layer chromatography (TLC) on 0.5 mm thick plates containing 10% silver nitrate. The plates were developed in petroleum ether/diethyl ether (40:60, v/v) and bands were detected by spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol and viewing under ultraviolet light. The methyl hydroxyesters were extracted from the silver ion plates by the Hill et al. procedure (8) and converted to TMS-derivatives as before (3).

Olive and soybean oils were randomized by stirring with 0.1% sodium methoxide at 60 C for 5 hr at 1 Torr. Randomized and natural oils were purified by chromatography through alumina before oxidation (9). After oxidation

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and reduction of the hydroperoxides, the oils were converted to methyl esters with 0.5% sodium methoxide in methanol and analyzed as before. Statistical analysis was according to SAS (10).

RESULTS AND DISCUSSION

The identity and purity of the TMS-derivatives of the methyl hydroxyesters produced from the oxidation of methyl oleate and linoleate have been established previously (3). To establish the identity of the TMS-derivative obtained from the oxidation of methyl linolenate, the hydroperoxides were reduced by iodide and the methyl hydroxyesters were isolated by TLC and silylated. GLC of the TMS-derivatives gave the 4 partly resolved peaks seen in Figure 1. These results are comparable to those reported by Frankel et al. (6) in their Figure 1B. An IR spectrum of the TMS-derivatives indicated the presence of TMS (845, 1250 cm^{-1}), methyl ester (1180, 1745 cm^{-1}), *cis,trans*-conjugated diene (950 cm^{-1}), and *trans,trans*-conjugated diene (988 cm^{-1}). The mass spectrum showed fragments at 183, 223, 311 and 351 and after hydrogenation at 131, 173, 187, 229, 259, 301, 315, 357 and 371 in agreement with the results of Frankel et al. (6).

GLC would not resolve the TMS-hydroxylinoleate and TMS-hydroxylinolenate, as a comparison of Figures 1 and 2 shows. Silver ion chromatography of the TMS-derivatives also failed to give satisfactory separation. Satisfactory resolution was obtained with methyl hydroxyesters on silver nitrate plates as shown in Figure 3. Only small amounts of hydroxyester (ca. 1 mg/spot) could be applied or resolution was lost.

Wong and Hammond (3) reported that the apparent yield by GLC of the TMS-derivatives was 63% from methyl oleate hydroperoxide and 40% from methyl linoleate hydroperoxide based on methyl heptadecanoate as an internal GLC standard. Pure methyl oleate, linoleate and linolenate were oxidized separately until they reached peroxide values of ca. 5, 10, 20 and 40 meq/kg. Samples were converted to TMS-derivatives, and GLC responses were measured compared with the response of methyl heptadecanoate. Results are in Figure 4. The apparent yields calculated from the slopes were 62, 44 and 17% for oleate, linoleate and linolenate hydroperoxide, respectively. Subsequent analyses were corrected by these factors. The reasons for these large correction factors are not certain. Wong and Hammond found that methyl hydroxystearate and methyl

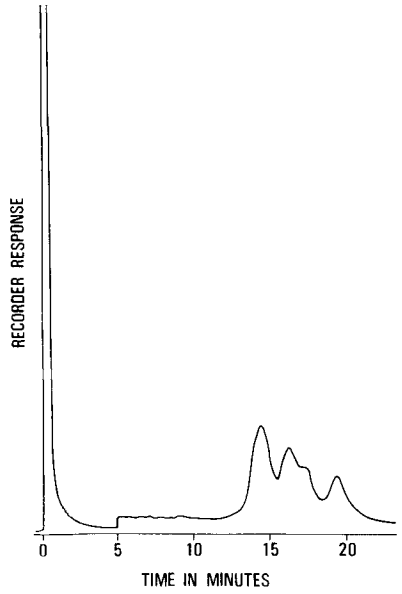

FIG. 1. A typical gas chromatogram of TMS-hydroxylinolenate on a 183-cm x .3-cm column packed with 10% OV 225 on Chromosorb W (HP). The carrier gas was nitrogen at 50 ml/min and the temperature was 180 C.

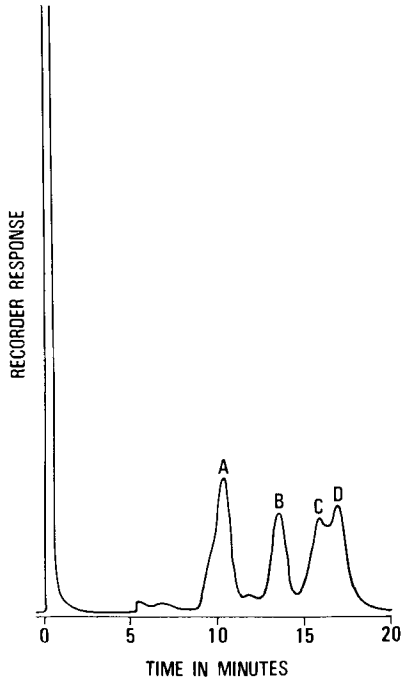

FIG. 2. A typical gas chromatogram of a mixture of TMS-hydroxyoleate and TMS-hydroxylinoleate. The conditions are the same as in Figure 1. Peak A is TMS-hydroxyoleate. Peaks B, C and D are TMS-hydroxylinoleate.

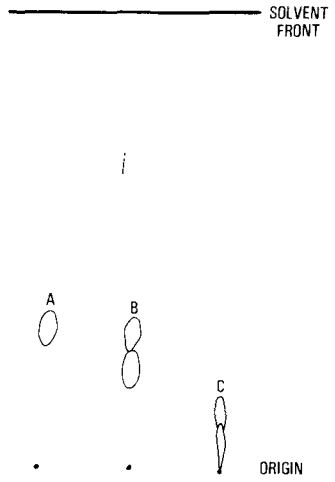

FIG. 3. TLC separation on silver nitrate-Silica Gel G of methyl hydroxyesters obtained from the reduction of hydroperoxides. The Silica Gel G contained 10% by weight of silver nitrate. The developing solvent was petroleum ether/diethyl ether (40:60, v/v). A is methyl hydroxyoleate, B methyl hydroxylinoleate and C methyl hydroxylinolenate.

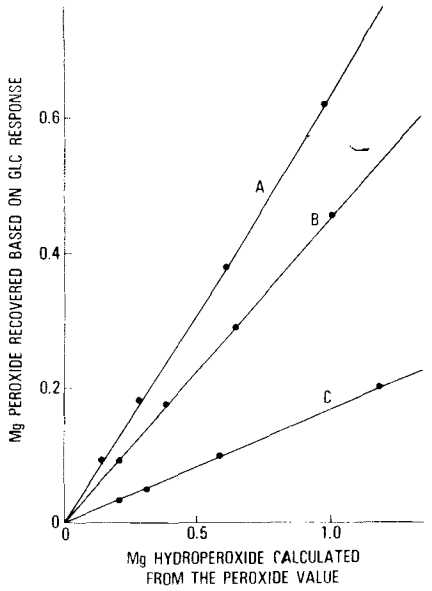

FIG. 4. The apparent recovery of TMS-hydroxy derivatives on the basis of GLC response compared with that calculated from the peroxide value. The slopes are a measure of the correction factors. A: methyl oleate; B: methyl linoleate; C: methyl linolenate.

ricinoleate both gave 78% yields and concluded that at least some of the correction factor was a result of the flame ionization detector's response to the silyl ethers. Some of the correction may arise from side reactions in the conversion of the hydroperoxides to TMS-hydroxyesters, but we have been unable to detect significant amounts of side products.

The analysis of the hydroperoxides in binary and trinary mixtures of the 3 esters is given in Table I. The data on oleate and linoleate are similar to those of Wong and Hammond (3), but include mixtures containing more oleate than they studied. In mixtures of 2 fatty acids the following propagation reactions can occur:

$R_1OO\cdot + R_1H \rightarrow R_1OOH + R_1\cdot$
with rate constant K_{11}
$R_1OO\cdot + R_2H \rightarrow R_1OOH + R_2\cdot$
with rate constant K_{12}
$R_2OO\cdot + R_2H \rightarrow R_2OOH + R_2\cdot$
with rate constant K_{22}
$R_2OO\cdot + R_1H \rightarrow R_2OOH + R_1\cdot$
with rate constant K_{21},

where R_1 refers to oleate and R_2 to linoleate. Fineman and Ross (11) have shown that

$$[(R_2OOH/R_1OOH) - 1]\ (R_1H/R_2H) = -(K_{11}/K_{12})\ (R_2OOH)/(R_1OOH)\ (R_1H/R_2H)^2 + K_{22}/K_{21}$$

and linear plots of the data allow K_{11}/K_{12} and K_{22}/K_{21} to be evaluated from slope and intercept. According to Russell (12), the various peroxy radicals are usually equally potent in abstracting hydrogen from RH and the rates of propagation are determined by the ease of abstraction of hydrogen from the various alkenes. If this is so $K_{11} \cong K_{21}$ and $K_{22} \cong K_{12}$ and $(K_{11}/K_{12})\ (K_{22}/K_{21}) \cong 1$. It follows also that, in trinary mixtures of oleate, linoleate and linolenate, data for oleate and linoleate should fall on the same line as the binary mixtures because it will be immaterial whether the peroxy radical is oleate, linoleate or linolenate, and all the hydroperoxide will accumulate according to the ease of hydrogen abstraction from the 3 alkenes. Such a plot for all the data in Table I is shown in Figure 5. The data from the trinary mixtures fall on about the same line as the binary data. $K_{22}/K_{21} = 8.38 \pm 0.54$ and $K_{11}/K_{12} = 0.0791 \pm 0.0070$. The product of the ratios is 0.66.

Not all of the points are equally reliable. When the amount of methyl oleate in the mixture is small, the amount of oleate hydroperoxide is very small and analytical error increases. This is particularly true because of a minor side product from linoleate oxidation that has the same retention time in the gas chromatograph as TMS–hydroxyoleate. This material is partly removed by TLC of the hydroxyesters, but when the TMS-hdyroxyoleate is small, this contaminant can cause

significant error. In Figure 5, these are the points in which the percentage of oleate hydroperoxide was less than 10%, many of which fall well below the straight line plots. If we omit these values, the data are fit best by a line with K_{22}/K_{21} = 10.15 ± 0.66 and K_{11}/K_{12} = 0.0944 ± 0.0069. The product of the ratios is 0.96.

It is not certain, however, that all the deviation shown in Fig 5 is caused by experimental error. Frankel et al. (6) reported data for oleate-linoleate mixtures that also have relatively large proportions of oleate hydroperoxide in mixtures containing low proportions of methyl oleate. Their analysis, based on mass spectrometry, should not be subject to the error we encountered. Moreover, to omit the mixtures that contained less than 10% oleate hydroperoxide, we had to omit all mixtures containing less than 60% methyl oleate.

If the peroxy radicals are all essentially equal in their ability to extract hydrogen from the alkene chains, then the best estimate of the relative ease of abstraction of hydrogen from linoleate compared with oleate would be the average of K_{22}/K_{21} and K_{12}/K_{11} which is 10.37. This agrees with the average relative oxidation rates calculated in Table I from the oleate-linoleate mixtures with large proportions of methyl oleate.

A Fineman-Ross plot of the linoleate-linolenate data of Table I is given in Figure 6. The data for the binary and trinary mixtures again fall on a single line, K_{33}/K_{32} is 1.623 ± 0.098 and K_{22}/K_{23} is 0.3927 ± 0.0110, where 3 refers to linolenate. The product of the 2 ratios is 0.64. If we average K_{33}/K_{32} and K_{23}/K_{22}, we find that the relative ease of linolenate oxidation compared with linoleate is 2.08. This

TABLE I

GLC Analysis of the Formation of Peroxide Types in Autoxidized Oleate, Linoleate and Linolenate Mixtures

Composition of methyl ester mixture					Peroxide %			Relative oxidation rate[a]	
18:1	18:2	18:3	P.V.	Recovery %[b]	Monoene	Diene	Triene	18:2/18:1	18:3/18:2
87.1	12.9		25.3	92.1	38.15	61.84		10.94	
77.31	22.69		33.3	91.2	25.64	74.36		9.9	
68.51	31.49		34.4	86.04	19.57	80.43		8.95	
57.98	42.02		41.5	93.6	13.04	86.98		9.2	
38.65	61.35		47.4	88.5	8.42	91.57		6.04	
19.73	80.27		57.4	103.7	5.55	94.45		4.2	
	89.66	10.34	55.8	89.3		80.14	19.86		2.14
	69.77	30.23	41.9	90.4		51.73	48.27		2.15
	49.16	50.84	34.5	89.4		30.98	69.02		2.15
	17.97	82.03	44.2	102.3		9.52	90.48		2.08
89.37	5.54	5.09	42.3	94.3	34.54	21.82	43.64	10.2	1.83
89.33	7.31	3.35	44.2	102.1	36.62	32.39	30.99	10.8	2.08
89.23	3.75	7.02	41.2	96.7	35.23	14.77	50.00	9.97	1.8
79.48	10.42	10.2	56.8	91.9	23.3	29.13	47.57	9.54	1.7
79.38	13.79	6.83	40.3	94.5	22.98	41.92	35.09	10.5	1.7
			55.3	89.7	21.9	43.81	34.29	11.5	1.6
79.22	7.14	13.64	49.8	87.1	20.2	18.31	61.48	10.05	1.75
			59.9	85.9	18.48	19.18	62.33	11.5	1.7
69.17	15.54	15.29	45.2	90.8	13.92	27.84	58.23	8.9	2.1
			64.5	89.7	11.54	30.12	58.33	11.6	1.96
69.11	20.70	10.19	39.9	86.4	14.35	42.58	43.06	9.9	2.05
			69.7	87.7	12.10	44.37	43.57	12.2	2
69.23	10.63	20.14	8.6	91.5	13.14	17.81	69.05	8.8	2.05
			53.9	87.6	11.91	20.30	67.69	11.1	1.8
5.33	88.9	5.77	52.6	105.2	---	86.03	13.97	---	2.5
13.27	70.84	15.89	36.5	104.4	1.97	67.83	30.19	6.5	1.98
22.60	52.12	25.28	65.9	92.4	2.39	52.56	45.05	9.5	1.8
33.67	30.17	36.16	41.6	88.3	3.09	27.99	68.92	10.1	2.05
44.01	12.32	43.67	40.4	91.4	4.26	12.52	83.22	10.5	1.9
14.92	15.11	69.97	51.7	89.4	1.56	10.16	88.28	6.4	1.9
22.80	25.75	51.45	35.5	93.4	2.41	17.91	79.68	6.6	2.23
33.26	36.12	30.61	35.9	86.2	4.53	30.74	64.72	11.2	1.8
42.44	46.42	11.14	37.9	90.2	6.06	62.88	31.06	9.5	2.06

[a] % R_2OOH x % R_1H/% R_1OOH x % R_2H or % R_3OOH x % R_2H/% R_2OOH x % R_3H, where R_1 is oleate, R_2 linoleate and R_3 linolenate.

[b] By GLC after applying correction factors.

agrees with the relative oxidation rate of 2.09 calculated from Table I.

A plot of the oleate-linolenate data from the trinary mixtures of Table I is given in Figure 7. These data show considerably more scatter. For all the points, K_{33}/K_{31} is 16.503 ± 0.918 and K_{11}/K_{13} is 0.337 ± 0.0049. If the points representing low percentages of oleate hydroperoxide are omitted, the values are 17.357 ± 1.438 and 0.0368 ± 0.0061, respectively. The products of the sets of ratios are 0.56 and 0.64, respectively. The averages of K_{33}/K_{31} and K_{13}/K_{11} indicate the linolenate oxidizes 23.09 and 22.23 times faster than oleate. If we multiply the estimated rates for (linoleate/oleate) x (linolenate/linoleate), this should also give the linolenate/oleate rate and is 10.37 x 2.08 = 21.57.

For linoleate-linolenate and oleate-linolenate mixtures, the product of slope and intercept are

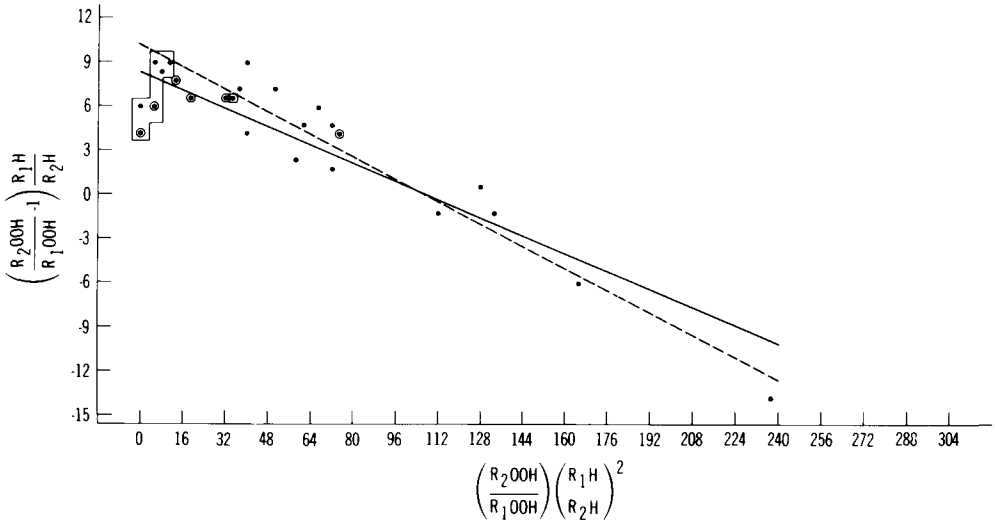

FIG. 5. A Fineman-Ross plot of the data for oleate and linoleate mixtures. The points with circles are from binary mixtures. The solid line is the best fit of all the points. The dashed line is the best fit of all points with more than 10% oleate hydroperoxide. The points with less than 10% oleate hydroperoxide are enclosed in rectangles. R_1 is oleate, R_2 linoleate.

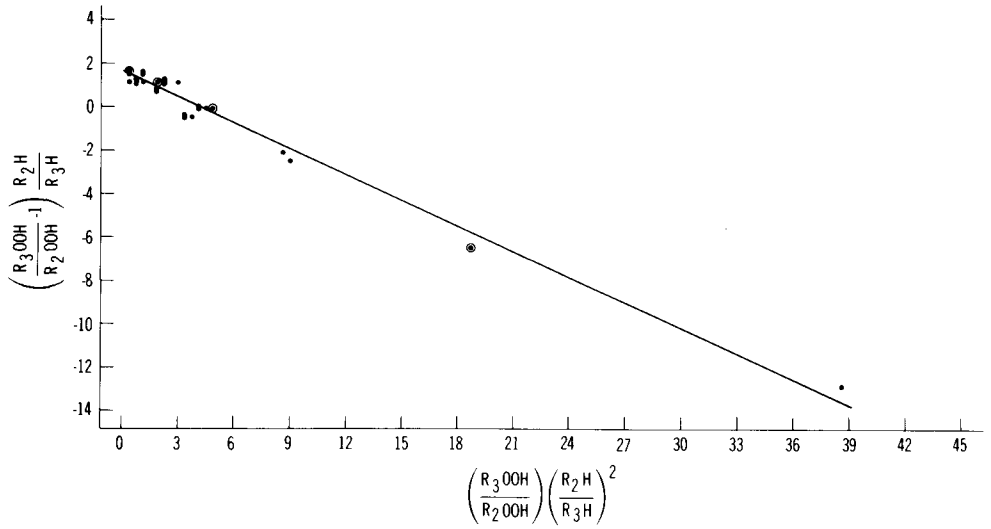

FIG. 6. A Fineman-Ross plot of the data for linoleate-linolenate mixtures. The circled points are from binary mixtures. R_2 is linoleate, R_3 linolenate.

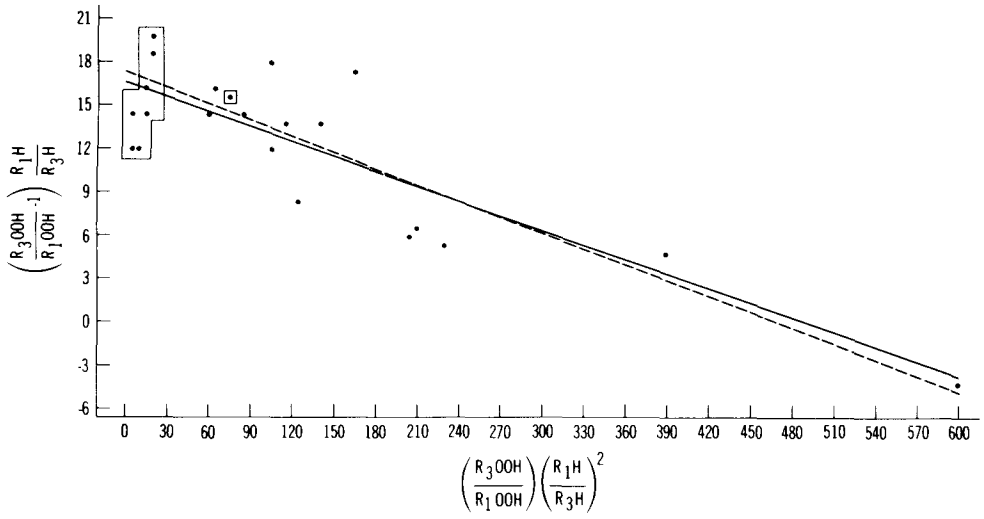

FIG. 7. A Fineman-Ross plot of the data for oleate-linolenate mixtures. The solid lines is the best fit for all the points with more than 10% oleate hydroperoxide. The points with less than 10% oleate hydroperoxide are enclosed in rectangles. R_1 is oleate, R_3 linolenate.

somewhat below 1. This may mean the peroxy radicals are not exactly equivalent or it may reflect some bias in the experimental data.

The relative oxidation rates we have found are very close to those obtained by the relative rates of oxidation of pure esters (1,2). This indicates that, in the oxidation of these pure esters, the rates of initiation must have been very similar. The relative rates of oxidation of these esters do not change appreiciably with extent of oxidation up to peroxide values of 70 meq/kg. Oxidations carried out at 21 C did not give results appreciably different from those at 28 C. The actual proportions of oleate and linoleate hydroperoxides we found are quite different from those reported by Frankel et al. (5,6), but this may be caused by the high temperatures and much higher peroxide values in their experiments. Such conditions are not conducive to quantitative recovery of hydroperoxides. Our results are much closer to those Frankel and Neff obtained for soybean oil (7), which were obtained under conditions more like the ones we used. But Frankel and Neff found in methyl ester mixtures prepared from soybean oil that at low peroxide values there was a relatively large proportion of 12-hydroxyoctadecanoate. The proportion of this isomer decreased as oxidation proceeded. We have noted no change with extent of oxidation in the proportions of products we isolated, but it is uncertain how a 12-hydroxydienoate or trienoate would behave in our separations.

Raghuveer and Hammond (13) suggested that glyceride structure can affect the relative rates of oxidation of acyl groups in a triglyceride. They suggested that acyl groups at the *sn*-1 and 3 positions should oxidize faster than those at *sn*-2. To study this, natural and randomized oils were oxidized and the analysis of the peroxides is given in Table II. The difference in the analysis of the randomized and unrandomized oils are the same within experimental error and similar to results expected on the basis of the methyl ester mixtures. However, this is not a very good test of Raghuveer and Hammond's theory. For example, we can calculate from the Fatemi and Hammond equations (14) that the unrandomized soybean oil used in this study should have an average composition on the *sn*-1, 3-positions of 26.60% oleate, 44.36% linoleate and 7.75% linolenate. Let us assume that the most extreme, and most easily detectible version of Raguhuveer and Hammond's proposal is that all the oxidation occurs on the *sn*-1, 3 positions and also that the relative rates of oxidation are those found here (1:10.3.21.6). Under these assumptions, the hydroperoxides should be found in the proportions 4.09% oleate, 70.20% linoleate and 25.72% linolenate. A similar calculation, assuming the soybean oil is completely randomized, yields values of 3.53%, 74.45% and 22.02%. The differences are not much greater than the experimental error of our method. Even so, the randomized oils oxidized about 3-4 times faster than the natural oils.

TABLE II

Analysis of the Hydroperoxides Formed during the Oxidation of Natural and Randomized Soybean and Olive Oils

Oil	% Fatty acid						% Peroxide			Relative oxidative rate	
	Sat	18:1	18:2	18:3	P.V.	% Recovery	Monoene	Diene	Triene	18:2/18:1	18:3/18:2
Soybean											
Natural	13.67	25.87	52.98	7.47	39.5	89.2	4.77	73.55	21.67	7.52	2.0
Random	13.61	25.44	53.24	7.71	36.1	87.4	4.57	72.87	22.54	7.7	2.13
Olive											
Natural	15.68	74.02	10.30	---	31.3	89.1	39.02	60.98	---	11.22	---
Random	16.20	73.16	10.64	---	40.6	91.6	40.50	59.50	---	10.09	---

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METHODS

Separation of Conjugated Bile Acids by Reverse Phase Thin Layer Chromatography

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ABSTRACT

Seven conjugated bile acids were separated by 1-dimensional reverse phase thin layer chromatography using ethanol/0.3 M calcium chloride/dimethylsulfoxide (25:25:2) as mobile phase.

INTRODUCTION

This report describes the complete separation of 7 major conjugated bile acids by reverse phase thin layer chromatography (RPTLC) in 1 dimension. Bile acids are present largely as conjugates with taurine or glycine. Thus, studies of bile acids and their metabolism should involve analysis of these without hydrolysis. Classical techniques used hydrolysis and derivatization for analysis by gas chromatography (1). This methodology is lengthy and results in loss of material and production of artifacts (2,3). Other methods such as paper, ion exchange and column chromatography, as well as electrophoresis, give inadequate separations. In recent years, a number of publications describing the use of thin layer chromatographic (TLC) separation of conjugated bile acids have appeared (4-7). These do not give complete resolution of all the individual conjugated bile acids with 1-dimensional development. The methods either used 2-dimensional development or did not completely resolve the dihydroxy conjugated bile acids. More recently, high performance liquid chromatography (HPLC) has been successful in separating the conjugated bile acids (8,9). Attempts to use the mobile phases reported for HPLC with reverse phase columns were unsuccessful when applied to RPTLC. Although the separations occurred, the resolution was such that scanning by densitometry was not possible. The Shaw and Elliott binary system (8) consisted of a 160:340 mixture of 2-propanol-8.8 mM potassium phosphate buffer (pH 2.5). This was modified to use the following: ethanol/0.3 M calcium chloride (pH 3)/dimethylsulfoxide (25:25:2). This new mobile phase gave separation of 7 conjugated bile acids as listed in Table I.

MATERIALS AND METHODS

All solvents were redistilled in glass. Ethanol (absolute) was obtained from Publicker Industries, Philadelphia, PA. Dimethyl sulfoxide was obtained from Aldrich Chemical Co., Milwaukee, WI. Calcium chloride was Baker reagent grade and was made up as 0.3 M in deionized distilled water and adjusted to pH 3 with hydrochloric acid. This was kept cold at 5 C until used. Whatman ($KC_{18}F$) reverse phase C_{18} plates were used. The mobile phase consisted of ethanol/0.3 M calcium chloride/dimethylsulfoxide (25:25:2). The bile acids, obtained from Steroloids, Wilton, NH, or Supelco, Bellefonte, PA, were made up as 100 ng/μl solutions in methanol. These were stored at 5 C when not in use. Human gall bladder bile samples obtained at surgery were diluted 1:100 in methanol. Bile samples obtained from rat bile fistulas were diluted 1:50 in water. Two- to 5-μl aliquots of these were applied directly to the origins of the reverse phase layers. These were developed in the mobile phase until the solvent front had moved to 5 mm from the upper edges, ca. 3 hr.

After drying at room temperature to evapo-

TABLE I

R_f Values for Conjugated Bile Acids on Reverse Phase Thin Layers

Bile acid	R_f
Taurocholic acid (TC)	0.38
Glycocholic acid (GC)	0.31
Taurochenodeoxycholic acid (TCDC)	0.26
Taurodeoxycholic acid (TDC)	0.23
Glycochenodeoxycholic acid (GCDC)	0.20
Glycodeoxycholic acid (CDC)	0.17
Glycolithocholic acid (GLC)	0.10

rate the solvent, the plates were heated in an oven at 170 C to remove residual solvent. Then the chromatograms were sprayed lightly with 10% sulfuric acid in ethanol followed by heating in an oven at 180 C for 2 min for development of characteristic fluorescence of the separated bile acids.

The chromatograms were then scanned on a Schoeffel Model 3000 spectrodensitometer in the transmission mode. The scanner was operated in the fluorescence manner with incident light of 360 nm. An A-74 Corning filter with cutoff at 400 nm was placed between the chromatogram and the viewing photomultiplier. This filter removed all light below 400 nm and enhanced the ratio between emitted signal and the noise.

RESULTS AND DISCUSSION

Table I gives the R_f values obtained for the 6 major conjugated bile acids found in human gall bladder bile. Visualization by eye under "long-wave" ultraviolet light shows clearly separated zones for the individual conjugated bile acids. The separation of the conjugated bile acids in rat bile obtained by fistula is shown along with the human bile acid chromatogram in Figure 1. Free bile acids did not migrate in the system described.

The major peaks appearing in the chromatogram of the human bile sample coincide in R_f with those of the standards. The chromatogram of the rat bile shows an entirely different pattern. Apparently, 2 of the major bile acids are taurocholic and taurochenodeoxycholic acids. This is in agreement with the results of Davis and Elliott (10) who used HPLC.

The method described here represents the first reported separation of all 6 of the major bile acids found in human bile in a single TLC system. The TLC method described differs from the other TLC systems so far reported in that reverse phase chromatography was used. Thus, it was possible to take advantage of mobile phases of higher polarity. However, use of the usual binary systems reported for HPLC separations with reverse phase was not successful in that the conjugated dihydroxy bile acids (GDC, GCDC and TDC, TCDC) did not separate well. The addition of dimethylsulfoxide to form the ternary system described changed the selectivity so that these 2 pairs were separated.

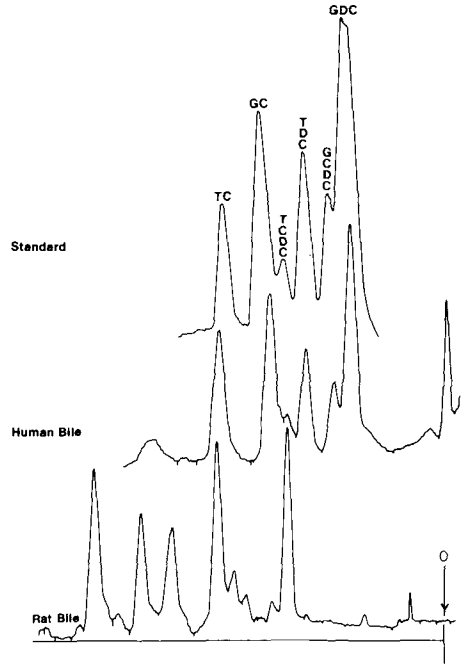

FIG. 1. Separation of conjugated bile acids in rat and human bile by reverse phase thin layer chromatography. See Table I for key to abbreviations. O = origin. The amount of standard was 100 ng for each conjugated bile acid.

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The Metabolism of Polyunsaturated Fatty Acids in Rat Sertoli and Germinal Cells

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ABSTRACT

The metabolism of [1-^{14}C]linoleic, [1-^{14}C]arachidonic and [3-^{14}C]docosa-4,7,10,13,16-pentaenoic acids was investigated after intratesticular injection of the labeled compounds and isolation of rat Sertoli and germinal cells. Following injection of either ^{14}C-linoleate or ^{14}C-arachidonate, the specific activity (sp act) of docosa-4,7,10,13,16-pentaenoic acid of Sertoli cells was greater than that of the germinal cells. The data suggest that the Sertoli cells are more active in the biosynthesis of the 22-carbon pentaene than the germinal cells. Differences between these 2 cell types were also noted in the distribution of the incorporated ^{14}C among the various lipid classes. Following intratesticular injection of ^{14}C-docosapentaenoic acid, a greater proportion of the recovered ^{14}C in Sertoli cells than in germinal cells was present in 20-carbon fatty acids, suggesting a greater activity in Sertoli cells in the metabolism of the pentaene. The major portion of the recovered ^{14}C in both cell types was present in triacylglycerols during early time periods and in phospholipids after 24 hr. The possibility of transfer of biosynthesized docosapentaenoic acid from Sertoli to germinal cells is discussed.

INTRODUCTION

Testicular lipids contain a large concentration of polyenoic acids which include, in addition to linoleic and arachidonic acids, members of the 22-carbon families (1). In testes of many species, including the rat, docosa-4,7,10,13,16-pentaenoic acid is present in large concentrations, whereas in others, including the human, the predominant 22-carbon polyene is docosa-4,7,10,13,16,19-hexaenoic acid. The importance of 22-carbon polyenes in the testis is suggested in that numerous factors which adversely affect the amount of the polyene in the testis also result in impaired spermatogenesis (2).

The association of 22-carbon polyenes with specific testicular cell types was suggested by Davis et al. (3). A decrease in the concentration of oleic acid and an increase in concentration of docosa-4,7,10,13,16-pentaenoic acid were observed in testes of rats at the time of appearance and maturation of spermatids. Agents or factors which depleted the testes of spermatids lowered the concentration of pentaene in the testis, whereas those affecting other cell types did not (4).

The development in recent years of methods of rapid cell separation has permitted studies to be performed on enriched fractions of specific cell types. Of considerable interest has been the finding in testes of rats that spermatids contained large quantities of docosa-4,7,10,13,16-pentaenoic acid (5) whereas spermatocytes (5) and Sertoli cells (6) contained much lower concentrations of this polyene. Lipids of isolated rat Leydig cells contain only small concentrations of 22-carbon polyenoic acids (A. Daniel, personal communication). Thus, it is evident that in rat testis docosapentaenoic acid is associated largely with the spermatids.

The investigations reported in this manuscript were concerned with the metabolism of polyenes in Sertoli and in germinal cells of rat testis. Of major interest was the conversion of linoleic and of arachidonic acids to docosa-4,7,10,13,16-pentaenoic acid and the incorporation of this polyene into the lipids of these cell types. In these studies, ^{14}C substrates were injected intratesticularly and the specific activities (sp act) of the metabolic products determined in isolated germinal and Sertoli cells after various time periods. The results indicated that the Sertoli cells were more active than the germinal cells in the conversion of linoleic and arachidonic acids to docosapentaenoic acid.

EXPERIMENTAL PROCEDURES

Sprague-Dawley rats were maintained on Purina laboratory chow from the time of weaning and were used for these studies when 65-150 days of age. Cell preparations were made from testes of individual rats. [1-^{14}C]-Linoleic acid (50 mCi/mmol) was obtained from New England Nuclear Corporation (Boston, MA), [1-^{14}C]arachidonic acid (50 mCi/mmol) was obtained from Amersham-Searle Corporation (Arlington Heights, IL) and [3-^{14}C]docosa-4,7,10,13,16-pentaenoic acid (0.44 μCi/mg) was biosynthesized from [1-^{14}C]arachidonic acid using cultures of *Euglena gracilis* (7). Each rat was injected in both testes with an albumin complex of either

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5 μCi of [1-^{14}C]linoleic acid, 2.5 μCi of [1-^{14}C]arachidonic acid or 0.5 μCi of [3-^{14}C]-docosapentaenoic acid. After specific intervals, the rats were killed, the testes quickly removed and immediately chilled in ice. Sertoli and germinal cells were prepared by the Steinberger et al. method (8), modified as described previously (6). The purity of the cell fractions was determined by criteria given by Meistrich et al. (9). The Sertoli cell fraction was at least 80% pure (spermatids were the major contaminant). Purity of the germinal cell fraction was at least 95%. The lipids of the cell fractions were extracted and hydrolyzed, and the released fatty acids were extracted using procedures that have been described previously (10,11). Fatty acids were methylated using the Metcalfe and Schmitz procedure (12). In some instances, fatty acid methyl esters were hydrogenated by the Farquhar et al. method (13). Classes of lipids were separated by thin layer chromatography (TLC) using the solvent system petroleum ether/diethyl ether/acetic acid (80:20:1, v/v). The silicic acid containing each lipid class was scraped into counting vials containing scintillation fluor and the radioactivity determined in a scintillation spectrometer. Methyl esters, separated by gas chromatography using a column packed with 10% SP-2340 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA), were collected using a fraction collector (Packard Instrument Co., Parnes Grove, IL), connected to the exit port of the thermal conductivity detector. The esters were trapped in glass cartridges packed with glass wool. The glass wool was subsequently transferred to scintillation vials containing scintillation fluid and the radioactivity determined in a scintillation spectrometer.

RESULTS

The incorporation of radioactivity into individual fatty acids of Sertoli and germinal cells following intratesticular injections of [1-^{14}C]linoleate is given in Table I. In both cell types, a considerable proportion of the ^{14}C recovered after 3 hr was still in linoleic acid. This amount decreased gradually with time with a concomitant increase in radioactivity in longer chain members of the linoleate family. A significant amount of the recovered radioactivity was in the palmitic acid. This was derived from ^{14}C-acetyl CoA produced by the β-oxidation of ^{14}C-linoleate. Small amounts of ^{14}C were also present in stearic and oleic acids (not included in Table I). For each time period studied, the proportion of ^{14}C in linoleic acid was slightly higher in germinal than in the Sertoli cell fraction. The proportion of ^{14}C in docosa-4,7,10,13,16-pentaenoic acid, on the other hand, was consistently higher in the Sertoli than in the germinal cell fraction. At all time periods, the sp act of linoleic acid in the germinal cells was about twice that of the Sertoli cells whereas the sp act of docosapenta-

TABLE I

The Distribution of ^{14}C in Fatty Acids of Isolated Rat Sertoli and Germinal Cells after Intratesticular Injection of [1-^{14}C]Linoleate

Time (hr)	Cell type	Recovered radioactivity (%)[a]					
		16:0[b]	18:2	20:3	20:4	22:4	22:5
3	Sertoli	7.8 ± 0.1[c]	67.6 ± 0.5	6.5 ± 0.9	10.2 ± 1.4	2.1 ± 0.5	2.0 ± 0.4
3	Germinal	8.0 ± 1.7	74.3 ± 2.3[d]	5.6 ± 0.7	8.3 ± 0.5[d]	0.9 ± 0.1	0.5 ± 0.1[e]
6	Sertoli	8.0 ± 1.9	52.2 ± 6.6	7.2 ± 2.9	16.9 ± 2.8	2.9 ± 1.4	4.5 ± 0.3
6	Germinal	10.3 ± 0.5	63.6 ± 1.6[d]	5.4 ± 1.4	16.0 ± 0.3	1.6 ± 0.1	1.2 ± 0.2[e]
12	Sertoli	10.0 ± 0.1	45.7 ± 0.7	7.9 ± 0.1	22.7 ± 0.7	5.4 ± 0.2	4.5 ± 0.2
12	Germinal	10.1 ± 1.3	63.3 ± 1.3[e]	7.4 ± 0.1	16.5 ± 0.1[d]	2.6 ± 0.7	1.9 ± 0.2[e]
24	Sertoli	8.7 ± 0.7	38.0 ± 1.3	7.4 ± 1.7	31.6 ± 0.9	4.5 ± 0.8	5.8 ± 0.8
24	Germinal	7.0 ± 0.4	53.8 ± 0.8[e]	7.7 ± 0.2	23.9 ± 0.7[d]	2.6 ± 0.2	2.9 ± 0.2[e]
48	Sertoli	7.7 ± 0.1	32.4 ± 2.8	7.3 ± 2.0	31.2 ± 4.6	6.2 ± 0.7	7.9 ± 1.7
48	Germinal	12.7 ± 0.9[e]	37.5 ± 2.8	6.1 ± 0.8	31.2 ± 2.1	3.8 ± 0.3	5.1 ± 0.4[d]

[a]Small amounts of radioactivity recovered in 18-carbon fatty acids are not shown.

[b]Number of carbons:number of double bonds.

[c]Mean ± standard error of the mean. There were 2 samples at 3, 6 and 12 hr, 3 at 24 hr, and 4 at 48 hr.

[d]Statistically significant difference from Sertoli cell value; $p < 0.05$.

[e]Statistically significant difference from Sertoli cell value; $p < 0.01$.

enoic acid in the Sertoli exceeded that of the germinal cell fraction (Fig. 1). The ratio of the sp act of docosapentaenoic acid in the Sertoli to that in the germinal cells was greatest at 3 hr (~9) and decreased with time such that at 48 hr after ^{14}C-linoleate injection the ratio was ca. 3. No consistent differences were seen between cell types in the sp act of the intermediates, eicosa-8,11,14-trienoic, arachidonic and docosa-7,10,13,16-tetraenoic acids.

The incorporation of ^{14}C into individual fatty acids of Sertoli and germinal cells following intratesticular injections of [1-^{14}C]-arachidonic acid is given in Table II. In both cell types, a greater proportion of the recovered ^{14}C was found in arachidonic acid (the injected substrate) than the proportion of ^{14}C found in linoleic acid after ^{14}C-linoleate injections. Moreover, less ^{14}C was incorporated into palmitic and 18-carbon fatty acids in both cell types following ^{14}C-arachidonic acid injection than occurred following ^{14}C-linoleate injections. Following ^{14}C-arachidonic acid administration, the Sertoli cells contained a greater proportion of ^{14}C in docosa-4,7,10,13,16-pentaenoic acid than the germinal cells at all time periods except 24 hr. The Sertoli cells also had a slightly greater proportion of ^{14}C in docosatetraenoic acid than the germinal cells, whereas the germinal cells contained a slightly greater proportion of ^{14}C at most time periods in arachidonic, palmitic and stearic acids. The sp act of the polyenoic acids are shown in Figure 2. The sp act of arachidonic acid was somewhat higher in the germinal than in the Sertoli cells at all time periods (not statistically significant at 1.5 hr). The sp act of docosa-7,10,13,16-tetraenoic acid was considerably greater in germinal than in Sertoli cells at 1.5, 3 and 6 hr but not at 24 hr. The greatest difference between the cell types, however, was in

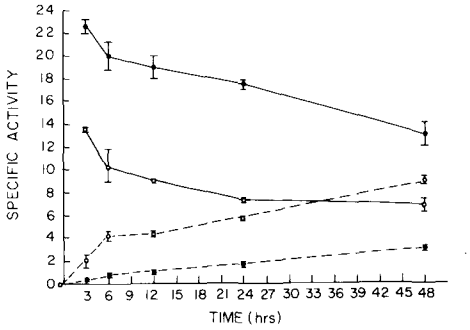

FIG. 1. The relative specific activities (sp act) of linoleic and docosa-4,7,10,13,16-pentaenoic acids in isolated rat Sertoli and germinal cells after intratesticular injection of [1-^{14}C]linoleate. Rats (60-150 days of age) were injected intratesticularly with [1-^{14}C]linoleate. Following the indicated time intervals, Sertoli and germinal cells were isolated from the testes as described under Experimental Procedures. Lipids of these cells were extracted and treated as described in Table I. Relative sp act are expressed as a percentage of total counts divided by the percentage of total fatty acids of the fatty acid in question. ●——● - linoleic acid, germinal cells; ○——○ - linoleic acid, Sertoli cells; ●----● - docosa-4,7,10,13,16-pentaenoic acid, germinal cells; ○----○ - docosa-4,7,10,13,16-pentaenoic acid, Sertoli cells.

TABLE II

The Distribution of ^{14}C in Fatty Acids of Isolated Rat Sertoli and Germinal Cells after Intratesticular Injection of [1-^{14}C] Arachidonate

Time (hr)	Cell type	Recovered radioactivity (%)[a]			
		16:0[b]	20:4	22:4	22:5
1.5	Sertoli	1.7 ± 0.3[c]	91.4 ± 0.6	3.7 ± 0.4	2.5 ± 0.7
1.5	Germinal	2.1 ± 0.1	93.6 ± 1.4	2.7 ± 0.7	0.3 ± 0.8[d]
3	Sertoli	2.4 ± 0.5	84.0 ± 1.3	7.6 ± 0.7	4.5 ± 0.4
3	Germinal	2.4 ± 0.2	89.1 ± 0.4[e]	5.2 ± 0.7[d]	1.1 ± 0.1[f]
6	Sertoli	2.3 ± 0.4	77.9 ± 3.3	7.8 ± 1.1	7.8 ± 1.5
6	Germinal	2.7 ± 0.7	89.6 ± 0.7[d]	5.1 ± 0.5[d]	1.5 ± 0.1[f]
24	Sertoli	2.4 ± 0.3	76.4 ± 2.5	12.6 ± 2.4	5.7 ± 0.4
24	Germinal	3.6 ± 0.1[e]	79.9 ± 1.5	7.8 ± 1.2	5.2 ± 0.4

[a]Small amounts of radioactivity recovered in 18-carbon fatty acids are not shown.

[b]Number of carbons:number of double bonds.

[c]Mean ± standard error of the mean. There were 2 samples at 1.5 hr, 4 at 3 and 6 hr and 3 at 24 hr.

[d]Statistically significant difference from Sertoli cell value; $p < 0.05$.

[e]Statistically significant difference from Sertoli cell value; $p < 0.01$.

[f]Statistically significant difference from Sertoli cell value; $p < 0.001$.

the sp act of docosa-4,7,10,13,16-pentaenoic acid, which was higher in the Sertoli than in the germinal cells for all time periods. The difference decreased with time but did not disappear even at 24 hr. Portions of the fatty acid extracts obtained from the cells of the ^{14}C-arachidonic acid-injected rats were hydrogenated and subjected to radio gas chromatographic analysis. The results were generally consistent with the values obtained with samples which were not hydrogenated (i.e., a smaller proportion in Sertoli cells of ^{14}C in the 20-carbon fatty acid fractions [from arachidonic acid] and a greater proportion in 22-carbon fatty acids).

The incorporation of ^{14}C into various lipid classes following injection of either ^{14}C-linoleate or ^{14}C-arachidonate is summarized in Table III. In both cell types, ^{14}C from either substrate was rapidly incorporated into complex lipids, and less than 10% of the recovered ^{14}C was in unesterified fatty acids. Following ^{14}C-linoleate administration, more ^{14}C was incorporated into phospholipids of germinal than of Sertoli cells at all time periods. The proportion of radioactivity recovered in triacylglycerols of Sertoli cells exceeded that in triacylglycerols of germinal cells. At the early time periods following ^{14}C-arachidonate administration, the distribution of ^{14}C in the various lipid classes was similar in the 2 cell types. After 24 hr, the germinal cells contained a greater proportion of ^{14}C in phospholipids and a lower proportion in triacylglycerols than the Sertoli cells.

Following ^{14}C docosapentaenoate injection, the ^{14}C recovered in the fatty acids of both cell types was primarily in the 22-carbon fraction (Table IV). A significant amount of activity was present also in 16- and 18- and 20-carbon fatty acids. At all time periods, the Sertoli cells contained a greater portion of their ^{14}C activity in the 20-carbon fraction than did the germinal cells.

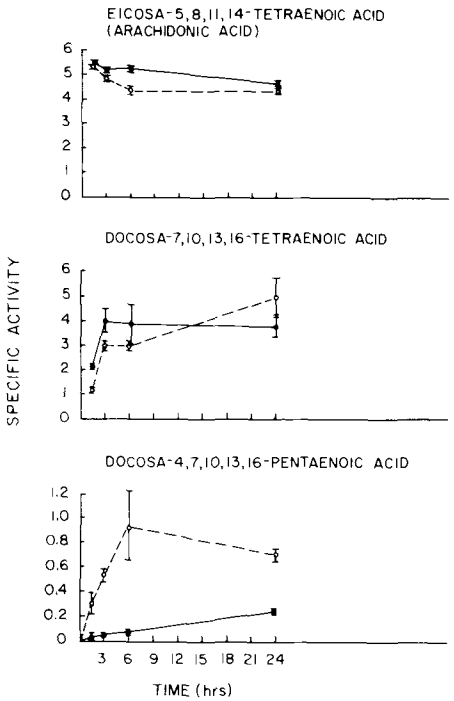

FIG. 2. The relative specific activities (sp act) of arachidonic, docosa-7,10,13,16-tetraenoic and docosa-4,7,10,13,16-pentaenoic acids in isolated rat Sertoli and germinal cells after intratesticular injection of [1-^{14}C] arachidonate. Rats (60-150 days of age) were injected intratesticularly with [1-^{14}C] arachidonic acid. Following the indicated time intervals, Sertoli and germinal cells were isolated from the testes as described under Experimental Procedures. Lipids of these cells were extracted and treated as described in Table I. Relative sp act are expressed as a percentage of total counts divided by the percentage of total fatty acids of the fatty acid in question. ●——● - germinal cells; o----o - Sertoli cells.

DISCUSSION

Previous studies revealed that the highest concentration of docosapentaenoic acid in rat testis was in the spermatid fraction (5,6). Although it has been established that the pentaene could be synthesized by the testis from intratesticularly administered linoleate or arachidonate (14), the site of biosynthesis in the testis is unknown. Following intratesticular injection of ^{14}C precursors of the pentaene in the experiments reported in this paper, the sp act of the pentaene in Sertoli cells was much higher than that of the pentaene in the germinal cells. The sp act of the injected substrate was higher, at all time intervals measured, in the germinal cells than in the Sertoli cells. It is therefore suggested that at least a portion of the synthesis is carried out by the Sertoli cells. From these data, it cannot be ascertained whether or not biosynthesis of the pentaene occurred also in germinal cells. It is possible that the polyene is synthesized largely in the Sertoli cells and then transported to the developing spermatid in some manner. Sertoli cells, although located primarily along the basement membrane, extend throughout the germinal epithelium, infiltrating between the germinal cells (15). Germinal cells are in close contact with Sertoli cells throughout this development, and during certain stages of their maturation they are embedded within Sertoli cell cytoplasm. It is during this time that Sertoli

TABLE III

The Distribution of ^{14}C in Lipid Fractions of Isolated Rat Sertoli and Germinal Cells after Intratesticular Injection of [1-^{14}C] Linoleate or [1-^{14}C] Arachidonate

Time (hr)	Cell type	Total ^{14}C recovered (%)[a]					
		[1-^{14}C] Linoleate			[1-^{14}C] Arachidonate		
		PL[b]	UFA	TG	PL	UFA	TG
1.5	Sertoli	---	---	---	47.4 ± 1.4[c]	8.6 ± 0.3	35.7 ± 1.3
1.5	Germinal	---	---	---	55.8 ± 2.9	3.1 ± 0.8	35.8 ± 2.6
3	Sertoli	53.0 ± 2.9	5.6 ± 0.1	31.0 ± 1.5	57.4 ± 2.0	5.3 ± 2.0	32.7 ± 1.4
3	Germinal	67.5 ± 0.7[d]	2.1 ± 0.1[f]	23.8 ± 0.3[e]	60.8 ± 2.9	3.2 ± 0.6	30.8 ± 1.8
6	Sertoli	56.2 ± 12.0	8.5 ± 6.0	27.3 ± 4.2	---	---	---
6	Germinal	75.0 ± 0.7	2.1 ± 0.2	17.8 ± 0.3[d]	---	---	---
12	Sertoli	55.6 ± 4.9	3.5 ± 0.4	29.5 ± 2.0	---	---	---
12	Germinal	72.3 ± 0.4[d]	1.1 ± 0.1[d]	22.8 ± 1.1[d]	---	---	---
24	Sertoli	60.2 ± 0.2	3.3 ± 0.3	27.4 ± 0.8	58.2 ± 4.6	5.2 ± 1.2	28.5 ± 2.9
24	Germinal	75.4 ± 0.4[f]	1.5 ± 0.1[e]	17.8 ± 0.2[f]	71.4 ± 1.3[d]	0.1 ± 0.1[e]	23.1 ± 1.9
48	Sertoli	66.6 ± 1.9	2.3 ± 0.9	20.3 ± 2.5	---	---	---
48	Germinal	77.4 ± 1.6[f]	1.8 ± 0.2	15.8 ± 1.2[d]	---	---	---

[a]Small amounts of radioactivity were recovered also in fractions migrating with monacylglycerols, cholesteryl esters and diacylglycerols.

[b]PL = phospholipids; UFA = unesterified fatty acids; TG = triacylglycerols.

[c]Mean ± standard error of the mean. The number of samples at each time period was the same as that in Table I for [1-^{14}C] linoleate and Table II for 1-^{14}C-arachidonate.

[d]Statistically significant difference from Sertoli cell value; $p < 0.05$.

[e]Statistically significant difference from Sertoli cell value; $p < 0.01$.

[f]Statistically significant difference from Sertoli cell value; $p < 0.001$.

TABLE IV

The Distribution of ^{14}C in Chemically Hydrogenated Derivatives of Fatty Acids of Isolated Rat Sertoli and Germinal Cells after Intratesticular Injection of [3-^{14}C] Docosapentaenoic Acid

Time (hr)	Cell type	Total ^{14}C recovered (%)				
		16:0[a]	18:0	20:0	22:0	24:0
1.5	Sertoli	3.2[b]	0.5	9.7	85.9	0.7
1.5	Germinal	2.2 ± 1.1[c]	0.6 ± 0.6	3.4 ± 1.7	92.8 ± 0.1	1.0 ± 1.0
3	Sertoli	1.2	0.4	15.0	82.4	1.0
3	Germinal	0.8	---	10.0	87.9	1.3
6	Sertoli	0.7	---	17.1	80.0	2.2
6	Germinal	0.9 ± 0.5	0.3 ± 0.3	9.3 ± 2.9	88.2 ± 3.8	1.2 ± 0.2
24	Sertoli	6.7 ± 2.1	2.6 ± 1.0	35.0 ± 3.0	52.4 ± 0.5	2.5 ± 0.6
24	Germinal	4.6 ± 1.5	1.6 ± 0.9	17.5 ± 3.8[d]	74.2 ± 4.5[d]	2.1 ± 0.5

[a]Number of carbons:number of double bonds.

[b]A single number indicates that the analysis was performed only once at that given time period.

[c]Mean ± standard error of the mean. Individual analyses were performed on all germinal cell preparations and on 2 24-hr and 3-hr Sertoli cell preparations. Portions of 2 Sertoli cell extracts obtained at 1.5 hr were pooled before analysis; portions of 2 6-hr Sertoli cell fractions were likewise pooled before analysis.

[d]Statistically significant difference from Sertoli cell value; $p < 0.05$.

cell lipid droplets have been observed, apparently being absorbed into spermatids (16,17). These lipid droplets may be enriched in docosapentaenoic acid. Sertoli cells contain a significant concentration of triacylglycerols in which is esterified a considerable quantity of docosapentaenoic acid (6). Triacylglycerols of spermatids also contain a considerable quantity of docosapentaenoic acid, much more than the triacylglycerols of spermatocytes (5). Following injection of [3-^{14}C]docosapentaenoic acid, most of the recovered ^{14}C in the total lipids was in triacylglycerols at the early time periods, and only at later time periods did it accumulate in phospholipids (data not shown). It therefore seems possible that triacylglycerols might serve as a vehicle for transporting the pentaene from Sertoli to germinal cells. Alternatively, the higher sp act of the Sertoli cell pentaene after ^{14}C-linoleate and ^{14}C-arachidonate administration might simply indicate a more active turnover rate of polyenoic acids than in the germinal cell. The experiment with injected labeled pentaene which resulted in more ^{14}C appearing in the 20-carbon fatty acid fraction of Sertoli than in germinal suggests this may be so. The fraction most likely resulted from arachidonic acid formed by retroconversion (biohydrogenation of one double bond followed by a 2-carbon cleavage) of the injected pentaene, a process known to be active in testicular tissue (18). Some ^{14}C-20-carbon fatty acids might have been synthesized from ^{14}C-acetate units formed by β-oxidation of the labeled pentaene. A considerable proportion of the radioactivity recovered 24 hr after ^{14}C-pentaene administration was in 16- and 18-carbon fatty acids in both cell types. Whatever the source of the ^{14}C in the 20-carbon fatty acids, it is apparent that the Sertoli cells were incorporating ^{14}C into this fraction at a rate far exceeding that of the germinal cells.

The importance of the Sertoli cells in the normal functioning of the testis is well recognized. Ultrastructural studies (19) suggest that in essential fatty acid deficiency the Sertoli cells are the earliest affected cell type in the testis. The lipid biochemistry of these cell types in essential fatty acid deficiency as well as in vitro biosynthesis of docosapentaenoic acid in individual cell types are currently being investigated.

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Effect of Plant Sterols, Fatty Acids and Lecithin on Cholesterol Absorption in vivo in the Rat

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ABSTRACT

The inhibitory effect of plant sterols, fatty acids and lecithin on cholesterol intestnal absorption was studied in the unanesthetized rat using a single pass perfusion technique. Bile was excluded from the perfused intestine. Cholesterol absorption did not change following the additions of cholestanol, cholestanone, lanosterol, stigmasterol and β-sitosterol. A 3-fold increase in the molarity of cholestanol and β-sitosterol or the separate additions of the saturated short and medium chain fatty acids, butyric and octanoic, also did not change cholesterol absorption. The unsaturated long chain fatty acids, oleic, linoleic, linolenic and arachidonic, inhibited cholesterol absorption. Lecithin additions at concentrations of 0.1-1.5 mM caused a progressive, dose-related inhibition of cholesterol absorption. The inhibitory effect of these agents on cholesterol absorption is likely to have been caused by changes in cholesterol solubility in the micelle and shifts in the partition coefficient of cholesterol away from the cell membrane to the micelle.

INTRODUCTION

Mammalian cholesterol is derived from endogenous synthesis or from dietary sources. Cholesterol from both sources undergoes extensive enterohepatic circulation and requires intestinal absorption for maintaining and regulating its total body pool.

Attempts to diminish the total body content of cholesterol by limiting dietary intake have failed (1) because the dietary contribution of cholesterol is small when compared to the amount synthesized endogenously (2). Interruption of or interference with the intestinal absorption of cholesterol by the use of resins to bind bile salts and cholesterol (3) or by the addition of cholesterol-like plant sterols to the diet (4) are possible approaches to decreasing body cholesterol content. The additions of fatty acids and lecithin to the intestinal contents have also been shown to be potential inhibitors of cholesterol absorption in vitro (5-7).

We studied the effect of plant sterols, fatty acids and lecithin in vivo on the absorption rate of cholesterol in the unanesthetized, restrained rat. The isolated perfused small bowel preparation we used enabled us to regulate the constituents of the intestinal contents and to measure the absorption of cholesterol without the interference of biliary secreted cholesterol.

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MATERIALS

(^{3}H 1α, 2α(n))Cholesterol with a specific activity (sp act) of 43 Ci/mmol (Amersham/Searle Co., Arlington Heights, IL) was used as a tracer compound. Nonradioactive cholesterol, lot number 74C-7440 (Sigma Chemical Co., St. Louis, MO), was of greater than 99% purity as ascertained by thin layer chromatography (TLC) on silica gel developed in cyclohexane-ethyl acetate (6:4) (8). Recrystallized sodium taurocholate (Calbiochem, Elk Grove, IL) had less than 1% impurities (9) as obtained. L-α-Lecithin, cholestanol, cholestanone, lanosterol, stigmasterol and β-sitosterol with purity greater than 98% were purchased from Serdary Research Laboratories, London, Ontario. Sodium dihydrogen phosphate and disodium hydrogen phosphate (J.T. Baker Chemical Co., Phillipsburg, NJ) were used as buffer components. The standard micellar perfusate was prepared in a Krebs phosphate buffer (10) which contained bile salts, sterols, fatty acids and cholesterol in concentrations as specified. The solution was irradiated with ultrasound (Artek Corp., Farmingdale, NY) for 5 min at 70 watts of power in order to form micelles. The micellar solution remained stable and optically clear for 24 hr at 20 C. The micellar solution containing ^{3}H-cholesterol was passed through a UM-2 filter (Amicon Corp., Lexington, MA) in order to test the partition of cholesterol between the micellar and the free monomeric forms. Since less than 3% of the radioactivity passed through the filter, we concluded that 97% or more of the cholesterol was dissolved within the micellar particles. The final osmolarity (11) of the solution varied between 280-310 mOsmol/ℓ.

METHODS

Male Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, MI) weighing 150-200 g were fed Purina Chow number 5012 (Check-R-Board, Novi, MI) and tap water ad libitum. The animals were kept in our laboratory for at least 2 wk prior to experimentation. Surgery and animal perfusion were performed in the morning hours (12). The rat was anesthetized with ether and an inflow catheter was introduced into the lumen of the small intestine 3 cm distally to the common bile duct's site of entry into the duodenum and was secured by an encircling ligature. A glass, L-shaped, outflow tube was then inserted into the distal ileum and secured by a ligature. The intestinal segment was returned to the peritoneal cavity which was closed surgically. The animal was allowed to awaken from the anesthetic and was placed in a Plexiglass restraining cage which allowed minimal mobility but prevented dislodgement of the catheters. A forced air heating device and a feed-back temperature controller monitored the animal's body temperature via a rectal probe and maintained the animal's body temperature at 37 C. After 1 hr of recuperation, the rats were infused with the micellar solution at a constant rate of 1 ml/min, using a syringe pump (Sage No. 351 Orion Research Inc., Cambridge, MA). The pumps were recalibrated frequently to ensure a constant infusion rate. The outflow through the glass cannula was collected in glass vials in separate aliquots every 20 min for a 3-hr period. The volume of each 20-min collection was measured, and concentration or dilution of each collection was detected by the addition of ^{14}C-inulin to the infusate. The final cholesterol concentration in each sample was corrected for fluid shifts (13). Water absorption in individual collections ranged from 1.9-6% with a mean value of 3%. The absorption rate of cholesterol was calculated by subtracting the amount of cholesterol collected out of the outflow tubing for each 20-min period from the total amount known to have been delivered into the inflow cannula over the 20-min period. The initial 20-min collection was always discarded. Cholesterol absorption rate during the 3-hr period of perfusion remained constant; the coefficient of variation between individual aliquots ranged from 8-13%. Absorption values were pooled from all the animals in each experimental series and a final mean ± S.E. value for absorption was calculated for each specific set of experimental conditions.

At the end of the experiment, the rats were killed by an overdose of ether and the intestinal segments were removed. After hanging overnight to dry with the glass cannula acting as a constant weight (4-5 g), the lengths of the segments were measured under conditions of constant stretch.

Radioactivity Determinations

100-μl aliquots of intestinal perfusate were pipetted into combusto-cones^{R} (Packard Instrument Co., Downers Grove, IL) containing filter paper. The samples were oxidized with a sample oxidizer (Tri-Carb Packard, Downers Grove, IL) to recover and separate the ^{3}H-cholesterol and ^{14}C-inulin. Radioactivity was assayed in a Beckman LS 250 liquid scintillation counter with automatic quench calibration at ambient temperature. Radioactive counts were carried to a counting error of ± 1%.

RESULTS

Adsorption of Cholesterol to Reservoir and Tubing

A perfusate solution consisting of the standard Krebs phosphate buffer at pH 7.2, 10 μM cholesterol and 10 mM sodium taurocholate was perfused through the tubing and glass cannula only to assess the degree of cholesterol adsorption (14). Less than 1% adsorption of cholesterol to the tubing and glass cannula was found.

Effect of Fatty Acid Addition to the Perfusate on Cholesterol Absorption

Short, medium and long chain fatty acids (1 mM) were added separately to the perfusate of several groups of animals. The standard perfusate contained constant amounts of cholesterol (10 μM) and sodium taurocholate (10 mM) and its pH was 7.2. The absorption rate of cholesterol in the presence of the various fatty acids was compared to its absorption rate in the absence of fatty acids. No difference in the absorption rate of cholesterol was noted following the additions of the saturated short and medium chain fatty acids, butyric and octanoic. All of the long chain unsaturated fatty acids decreased cholesterol absorption but the differences between the inhibitory effects of these fatty acids were not statistically significant (Table I).

Influence of Lecithin on Cholesterol Absorption

Solutions containing 10 μM cholesterol, 10 mM taurocholate in the Krebs phosphate buffer and progressively higher concentrations of lecithin were infused into groups of rats to ascertain the influence of lecithin on cholesterol absorption. Fatty acids or monoglycerides were not added to the perfusate in this series of

experiments. Lecithin addition at 0.1 mM concentration did not change the absorption rate of cholesterol when compared to baseline experiments. Lecithin additions at concentrations from 0.5 to 1.5 mM caused a progressive inhibition of cholesterol absorption (Table II).

Effect of Other Sterols on Cholesterol Absorption

The effect of various sterols on cholesterol absorption was studied using equimolar concentrations of cholesterol and a variety of sterols. The concentration of cholesterol was reduced to 1 μM because of its insolubility in the micellar solution at higher concentrations following the additions of the other sterols. None of the compounds studied had any effect on cholesterol absorption (Table III). Tripling the concentration of the 2 sterols, cholestanol and β-sitosterol, also had no inhibitory effect on cholesterol absorption (Table IV).

DISCUSSION

Studies in man have demonstrated that some of the plant sterols diminish intestinal absorption of cholesterol (4,15). However, assessment of cholesterol absorption in man is imprecise because of the large amount of endogenously synthesized cholesterol which is secreted into the small bowel. The multiplicity of methods

TABLE I

Effect of Fatty Acids Addition on 10 μM Cholesterol Absorption

Fatty acid	No. animals	Water absorption[a]	Cholesterol absorption[b]	p[c]
(1.0 mM)		(%)	(nmol/hr/100 cm)	
None	6	1.9 ± .47	238 ± 23	---
Butyric	4	1.9 ± .13	231 ± 3	> 0.05
Octanoic	3	1.6 ± .65	242 ± 16	> 0.05
Oleic	6	1.7 ± .20	163 ± 8	< 0.01
Linoleic	5	1.6 ± .30	128 ± 12	< 0.01
Linolenic	6	2.8 ± .45	140 ± 8	< 0.01
Arachidonic	4	2.5 ± .34	154 ± 7	< 0.01

[a]Values are mean ± SE of water absorption as calculated from ^{14}C-inulin concentration changes.

[b]Values are mean ± SE of cholesterol absorption rate from all animals at each set of experimental conditions. Eight separate values were obtained from each animal.

[c]Comparison between absorption rate in the absence of fatty acids to cholesterol absorption following their addition. Analysis of variance and t-test were used for statistical comparison.

TABLE II

Effect of Lecithin Addition on 10 μM Cholesterol Absorption

Lecithin	No. animals	Water absorption[a]	Cholesterol absorption[b]	p[c]
(mM)		(%)	(nmol/hr/100 cm)	
None	6	1.9 ± .47	238 ± 23	---
0.1	4	6.0 ± 1.2	214 ± 22	> 0.05
0.5	4	4.2 ± 1.1	134 ± 12	< 0.01
1.0	7	3.6 ± .90	115 ± 10	< 0.01
1.5	3	6.0 ± 1.7	109 ± 6	< 0.01

[a]Values are mean ± SE of water absorption as calculated from ^{14}C-inulin concentration changes.

[b]Values are mean ± SE of cholesterol absorption rate from all animals at each set of experimental conditions. Eight separate values were obtained from each animal.

[c]Comparison between absorption rate in the absence of lecithin to cholesterol absorption following the addition of lecithin. Analysis of variance and t-test were used for statistical comparison.

which have been proposed for the assessment of cholesterol absorption in man attests to the fact that none are unequivocally effective in overcoming the methodological problems involved (2, 16-19).

We studied the effects of a variety of sterols, fatty acids and lecithin on the intestinal absorption of cholesterol in vivo in the rat. By excluding biliary secretions from the perfused intestinal segment, we ensured that biliary cholesterol did not interfere with the measurement of cholesterol absorption.

We performed separate cholesterol absorption experiments following the additions of cholestanol, cholestanone, lanosterol, stigmasterol and β-sitosterol to the infusate. The basal rate of cholesterol absorption by the small bowel (20.9 ± 1.0 nmol/hr/100 cm) did not change following the equimolar additions of the sterols already discussed (Table III). Since no change in the rate of cholesterol absorption occurred in this series of experiments, we measured cholesterol absorption following the additions of cholestanol and β-sitosterol in 3 times the molar concentration of cholesterol. No change in cholesterol absorption rates was seen in these experiments, either (Table IV). When attempts were made to add other sterols

TABLE III

Effect of Other Sterols on 1 μM Cholesterol Absorption

Added sterol	No. animals	Water absorption[a]	Cholesterol absorption[b]	p[c]
(1 μM)		(%)	(nmol/hr/100 cm)	
None	6	4.5 ± 1.0	20.9 ± 1.0	---
Cholestanol	4	5.9 ± .99	21.3 ± 1.0	> 0.05
Cholestanone	4	2.4 ± .98	22.5 ± 1.6	> 0.05
Lanosterol	4	5.0 ± 1.8	20.2 ± 1.8	> 0.05
Stigmasterol	4	3.5 ± 1.7	23.2 ± 1.7	> 0.05
β-Sitosterol	4	3.8 ± .53	21.7 ± 1.0	> 0.05

[a]Values are mean ± SE of water absorption as calculated from ^{14}C-inulin concentration changes.

[b]Values are mean ± SE of cholesterol absorption rate from all animals at each set of experimental conditions. Eight separate values were obtained from each animal.

[c]Comparison between absorption rate in the absence of sterols to cholesterol absorption following their addition. Analysis of variance and t-test were used for statistical comparison.

TABLE IV

Influence of Other Sterol Concentration on 1 μM Cholesterol Absorption

Added sterol	No. animals	Water absorption[a]	Cholesterol absorption[b]	p[c]
(μM)		(%)	(nmol/hr/100 cm)	
None	6	4.5 ± 1.0	20.9 ± 1.0	---
Cholestanol (1)	4	5.9 ± .99	21.3 ± 1.0	> 0.05
Cholestanol (3)	4	2.3 ± .33	20.3 ± 0.4	> 0.05
β-Sitosterol (1)	4	3.8 ± .53	21.7 ± 1.0	> 0.05
β-Sitosterol (3)	3	4.7 ± 1.9	23.4 ± 1.1	> 0.05

[a]Values are mean ± SE of water absorption as calculated from ^{14}C-inulin concentration changes.

[b]Values are mean ± SE of cholesterol absorption rate from all animals at each set of experimental conditions. Eight separate values were obtained from each animal.

[c]Comparison between absorption rate in the absence of sterols to cholesterol absorption following their addition in 1 or 3 μM concentrations. Analysis of variance and t-test were used for statistical comparison.

in higher concentrations to the perfusate, micellar solubilization of cholesterol was disrupted and both cholesterol and the other sterols precipitated out of the perfusate.

Lees et al. used a cholesterol balance method to measure cholesterol absorption in patients with hypercholesterolemia and found that β-sitosterol inhibited cholesterol absorption (15). The discrepancy between our results and theirs may simply reflect species differences in cholesterol absorption. On the other hand, the differences may have resulted from the technique used in our experimental preparation which enabled us to measure cholesterol absorption without the interference of endogenous cholesterol secreted into the intestinal lumen.

In the next series of experiments, we tested the influence of fatty acids on the absorption of cholesterol. The additions of short and medium chain fatty acids to the perfusate did not change the rate of cholesterol absorption (Table I). Since the short and medium chain fatty acids are known to have solubility characteristics and absorptive patterns which are different from those of cholesterol-like compounds (20), these experimental findings were not unexpected. On the other hand, the additions of the long chain monounsaturated fatty acid, oleic, and the polyunsaturated fatty acids, linoleic, linolenic and arachidonic, resulted in a significant decrease in the amount of cholesterol absorbed (Table I). Lecithin was found to be the most effective inhibitor of cholesterol absorption. As the lecithin concentration in the perfusate was increased from 0.5 to 1.5 mM, a dose-dependent inhibition in cholesterol absorption was observed (Table II). Lecithin addition to the perfusate in 1.5 mM concentration resulted in more than 50% inhibition in cholesterol absorption rate when compared to baseline values (Table II).

Multiple mechanisms could be responsible for the inhibitory effect of the unsaturated fatty acids and lecithin on cholesterol absorption. The rate of transfer of cholesterol from the micelles to the cell membrane would depend on the partitioning of cholesterol between the micelle and the cell membrane (21,22). The addition of the unsaturated fatty acids or lecithin to the micellar incubation medium would enhance the solubility of cholesterol in the micelles and shift the partition coefficient of cholesterol away from the cell membrane to the micelle (23). This shift in partitioning of cholesterol would slow its absorption from the micellar perfusate and would result in the observed decrease in the absorption rate of cholesterol by the small bowel (Tables I and II). Lecithin has been recently shown to diminish the intestinal absorption of oleic acid from a mixed micellar solution in vivo by decreasing its monomer concentration at the absorptive cell membrane (24). This observation supports our proposed mechanism of lecithin inhibition of cholesterol absorption by increasing its micellar solubility and decreasing its monomer concentration. Additionally, the fatty acids and lecithin would increase the physical size of the micellar particles (25). The larger-sized micelles would diffuse towards the absorptive cell membrane of the enterocytes at a rate lower than that of the smaller micelles containing cholesterol only. The lower diffusion rate of the larger-sized micelles would also add to the decrease in the absorption rate of cholesterol observed in this series of experiments (Tables I and II).

The unstirred water layer is a known barrier to diffusion of micellar particles towards the absorptive cell membrane. It has been demonstrated to be a rate-limiting step in the uptake of cholesterol in vitro in the small intestine (22). Therefore, the inhibitory effect of the unsaturated fatty acids and lecithin on cholesterol absorption (Tables I and II) is undoubtedly accentuated by the resistance of the unstirred layer to diffusion of the larger-sized micellar particles.

Finally, lecithin and the unsaturated fatty acids may decrease cholesterol absorption by increasing the negative surface charge of the micellar particles. Both the micellar surface (26) and the luminal surface of the enterocytes (27) are negatively charged. The resistance to the diffusion of the micellar particles increases as the particles approach the absorptive cell membrane (28); thus, the absorption of substances dissolved within the micelles is retarded if the negative surface charge of the micelles is increased. Since the pKa of long chain fatty acids in sodium taurocholate micelles is 6.5 (29), a substantial portion of the fatty acids or lecithin would be in the charged state at the pH range used in this series of experiments. The additions of fatty acids or lecithin to the perfusion solution would increase the negative charge of the micellar surface—an increase which would interfere with diffusion of the micellar particles towards the negatively charged absorptive cell membranes. It is most probable that a combination of all of these factors described plays a role in decreasing the absorption rate of cholesterol following the additions of unsaturated fatty acids or lecithin (Tables I and II).

This series of experiments was performed in the absence of monoglycerides in the perfusate

solution. Monoglycerides were deleted from the perfusate in order to delineate the influence of fatty acids on cholesterol absorption without interaction with monoglycerides. Monoglycerides have been demonstrated to increase the absorption of cholesterol by enhancing its solubility in the micelles and preventing its precipitation out of the perfusate (30,31). Normally, monoglycerides are present within the micellar particles following lipid meals. Therefore, the influence of fatty acids, sterols and lecithin on cholesterol absorption will have to be evaluated in the presence of monoglycerides as well before these data can be extrapolated to cholesterol absorption under postprandial conditions.

If the methodological problems of studying human cholesterol absorption can be resolved, further experiments using varied concentrations of unsaturated fatty acids or lecithin as dietary additives should be tried in an attempt to disrupt intestinal cholesterol absorption. It must be emphasized, however, that unsaturated fatty acids and lecithin have been demonstrated to inhibit the intestinal absorption of fat-soluble vitamins in the rat (32-34). Therefore, their therapeutic trials in man should be carefully monitored for possible induction of fat-soluble vitamin deficiencies.

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Regulation of Lung Surfactant Cholesterol Metabolism by Serum Lipoproteins

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ABSTRACT

The isolated perfused rat lung was used as an experimental model in the study of the lipoprotein regulation of surfactant cholesterol metabolism. Addition of low density lipoproteins (LDL) to the perfusion medium at a cholesterol concentration of 0.5 mM had no inhibitory effect on [1-^{14}C]-acetate incorporation into cholesterol of either the surfactant or residual fractions. Increasing the concentration of cholesterol in the medium to 2.5 mM resulted in significant inhibition of incorporation into cholesterol of both fractions. A similar inhibition resulted when lungs were perfused with 2.5 mM cholesterol in the form of high density lipoproteins (HDL). No inhibition of fatty acid synthesis, measured as incorporation into cholesteryl esters, was observed. The rate of uptake by perfused lung of cholesterol from both high and low density lipoproteins was similar. Competitive binding studies with ^{125}I-labeled lipoproteins indicated the existence of lung receptors for both classes of lipoprotein. The rate of uptake of the apoprotein moiety of low density lipoproteins was significantly greater than that of high density lipoproteins. These data suggest that lung cholesterol metabolism may be subject to regulation by both low and high density serum lipoproteins.

INTRODUCTION

The surface tension constancy of lung alveoli is maintained by a lipoprotein complex termed pulmonary surfactant, of which dipalmitoylphosphatidylcholine is the principal lipid component (1-3). Cholesterol is next most abundant, comprising 8-14% of the total surfactant lipid (3-5). The lung has been shown capable of endogenous cholesterol synthesis (6,7); elevated levels of dietary cholesterol cause partial inhibition of that synthesis (8).

In vivo studies concerning possible regulation of lung cholesterol metabolism by serum lipoproteins have demonstrated uptake by rat lung of very low density lipoproteins (VLDL) and high density lipoproteins (HDL) (9,10), whereas in vitro experiments have shown both uptake and proteolysis of VLDL by perfused rat lung (11,12). Intravenous injection of unfractionated rat or human lipoproteins into rats made hypocholesterolemic by treatment with 4-aminopyrazolo [3,4-d] pyrimidine produced significant inhibition of cholesterol synthesis (13).

Results of experiments from our laboratory indicate that, while the isolated perfused rat lung is capable of endogenous cholesterol synthesis, use of exogenous lipoprotein cholesterol is the principal mechanism employed in the production of surfactant cholesterol (14). These studies investigate further the regulation of surfactant cholesterol production by specific serum lipoprotein classes.

METHODS

Normal-fed male Wistar strain rats (150-250 g) were used in these experiments. Rat lungs were perfused as previously described by Godinez and Longmore (15) except that perfusate vol was 40 ml. A 90% O_2/10% CO_2 gas mixture was used both for ventilation of the lung and oxygenation of the circulating perfusion medium, which consisted of Krebs Ringer bicarbonate buffer (pH 7.35) containing 3% bovine serum albumin and 5.6 mM glucose.

Water soluble isotopes were dried under N_2, redissolved in an aliquot of perfusion medium and then added to the recirculating perfusion medium. [1,2-^{3}H] Cholesterol was dried under N_2 and then adsorbed to serum lipoproteins by incubation at room temperature for 30-60 min prior to addition to the perfusion medium. Centrifugation of the incubated lipoproteins on sucrose density gradients indicated that greater than 90% of the radioactive cholesterol was bound to lipoproteins by this procedure. Following each perfusion, the lung was post-perfused using a 3-way valve with ca. 15 ml of unlabeled medium in order to remove the majority of labeled medium from the lung vasculature.

Endogenous cholesterol synthesis was measured by lung perfusions with medium containing 0.1 mM sodium acetate, [1-^{14}C]-acetate (30 μCi) and varying concentrations of seum lipoproteins. In these experiments, the lung was preperfused with lipoproteins for 10 min prior to the addition of the radioactively labeled acetate. Uptake of lipoprotein cholesterol was measured by lung perfusions with medium containing 1.0 mM lipoprotein cholesterol and [1,2-^{3}H] cholesterol (25 μCi). Specific activities of [1-^{14}C] acetate and [1,2-^{3}H]-

cholesterol were determined from samples of perfusion medium at zero time. For these substrates, incorporation data from all experiments were normalized to 1 x 10^6 dpm/μmol substrate to correct for differences in initial medium activity. Binding and uptake of lipoprotein protein was measured by perfusion with medium containing ^{125}I-labeled lipoproteins (minimum of 15,000 cpm/mg), with dextran substituted for bovine serum albumin.

In measuring competitive lipoprotein binding, the lung was perfused with ^{125}I-labeled lipoproteins for 5 min and then with unlabeled, unrecirculated medium for an additional 5 min to remove unbound lipoproteins. At this point, a tissue sample was taken, representative of bound lipoproteins.

Lipoprotein fractions were isolated by manganese chloride-dextran sulfate precipitation from pooled normal human serum according to the Burstein et al. method (16), with a minor modification. Instead of floating or pelleting the lipoproteins to either the top or bottom of the ultracentrifuge tube, LDL were cushioned above a layer of potassium bromide of density 1.074, and HDL floated beneath a layer of similar density. This aided in subsequent dispersion of the isolated lipoproteins into aqueous solution. Contaminating salts were removed by dialysis against 20 mM Tris-Cl (pH 7.7). The purity of the lipoprotein fractions was judged by electrophoresis on agarose gels. The LDL fraction appeared essentially homogeneous whereas the HDL fraction at times showed 5-10% contamination by LDL. Similar results were obtained whether the gels were stained for lipid (Fat Red 7B) or protein (Coomassie Brilliant Blue).

Radioiodination of LDL and HDL fractions was by the iodine monochloride method of McFarlane (17). Unreacted Na^{125}I was removed by extensive dialysis vs 20 mM Tris-Cl buffer (pH 7.7). Agarose gel electrophoresis of the iodinated lipoproteins indicated no significant change in migration; the radioactivity was associated only with the respective lipoprotein bands. About 91% of the LDL radioactivity and 98% of the HDL radioactivity was trichloroacetic acid precipitable, of which 9% and 2%, respectively, were extractable by chloroform/methanol (2:1).

The surfactant and residual fractions of rat lung were separated according to the Frosolono et al. method (2) as modified by Sanders and Longmore (18). The residual fraction obtained is comprised of all lung tissue fractions with the exception of the surfactant fraction. Total lung protein was determined by the Lowry et al. method (19). Lung fractions were extracted according to the Folch et al. method (20) as adapted by Radin (21). Lipid fractions were separated by silicic acid column chromatography, neutral lipids being eluted with 50 ml of chloroform. The various neutral lipids were further separated by thin layer chromatography (TLC) on plates of Silica Gel G using the solvent system described by Hass and Longmore (14). Following the visualization with iodine vapors, the areas of the gel containing cholesterol and cholesteryl esters were scraped into test tubes and extracted 3 times with chloroform/methanol (2:1). Aliquots were dried under N_2, then assayed for cholesterol by the Allain et al. enzymatic method (22), using a commercially available kit.

Quantitation of ^{3}H and ^{14}C radioactivity was obtained by liquid scintillation counting of aliquots of perfusion medium and lipid samples; the channels ratio method was employed to determine the efficiency of counting. ^{125}I was measured on a Packard Tri-Carb γ-counter. Results of experiments measuring cholesterol synthesis and uptake of lipoprotein cholesterol are expressed as the mean plus or minus the standard error of the mean, with the Student t-test used in determination of p values. Each point on the y-axis of figures illustrating competitive lipoprotein binding represents the mean of at least 3 samples, whereas all other points in figures are representative of single samples. Radioactive isotopes were purchased from New England Nuclear Corp. (Boston, MA). Materials for cholesterol assays were purchased from Boehringer-Mannheim Corp. (Indianapolis, IN).

RESULTS

Endogenous Cholesterol Synthesis

Initially, it was determined that the normal serum cholesterol concentration of rats used in this study was 1.7 mM. Since an occasional 5-10% contamination of the HDL fraction with LDL had been observed, experiments were performed in order to determine whether such low levels of LDL might inhibit endogenous cholesterol synthesis from acetate (Table I). Compared to control experiments in which lungs were perfused with lipoprotein-free medium, perfusion with medium containing 0.5 mM LDL cholesterol resulted in no inhibition of acetate incorporation into cholesterol of either the surfactant or residual fractions. However, increasing the medium concentration of LDL cholesterol to 2.5 mM resulted in significant inhibition of cholesterol synthesis. Likewise, perfusion with 2.5 mM HDL cholesterol produced a similar inhibition.

While it seemed highly unlikely, it was possible that the addition of lipoproteins to the perfusion medium may have resulted in sufficient perturbation of the intracellular acetyl-CoA pool to cause the decreased incorporation of [1-^{14}C]acetate into lung cholesterol. In preliminary control experiments, it was found that greater than 80% of the radioactivity incorporated into cholesteryl esters was found in the fatty acyl moiety. Since others have reported that within a few hours of cholesterol administration [1-^{14}C]acetate is incorporated principally into the fatty acyl moieties of lipids (23) and since modulation of acyl coenzyme A:cholesterol acyltransferase activity has been reported only after extended cholesterol exposure (24,25), incorporation into cholesteryl esters was used as a measure of fatty acid synthesis by the lung. As shown in Table II, in no case did perfusion with lipoproteins result in decreased incorporation into cholesteryl esters. Hence, the observed inhibition of acetate incorporation into unesterified cholesterol is not likely caused by alterations of the intracellular acetyl-CoA pool.

Uptake of Lipoprotein Cholesterol

That perfusion with both LDL and HDL inhibited endogenous cholesterol synthesis suggested cholesterol of both classes of lipoprotein were taken up by the perfused lung at relatively similar rates. The results of experiments testing this possibility are found in Table III. As anticipated, uptake of ^{3}H-cholesterol from both LDL and HDL into the surfactant and residual fractions of the lung was quite similar and only somewhat higher than that found earlier for a mixed LDL/VLDL fraction (14).

Competitive Lipoprotein Binding

Uptake processes specific for LDL and HDL would require that the lung have specific receptors for both classes of lipoproteins. Competitive binding studies were carried out to investigate whether this was the case. In Figure

TABLE I

Effect of Lipoproteins on [1-^{14}C] Acetate Incorporation into Cholesterol of Surfactant and Residual Fractions

Medium concentration	Rate of acetate incorporation into cholesterol	
	Surfactant	Residual
	pmol/mg protein/hr ± SEM[a]	
Control (0)	1.06 ± 0.16 (7)[b]	7.72 ± 1.17 (7)
0.5 mM LDL cholesterol	1.31 ± 0.20 (4)	6.78 ± 1.39 (4)
2.5 mM LDL cholesterol	0.55 ± 0.07 (3)[c]	3.20 ± 0.71 (3)[d]
2.5 mM HDL cholesterol	0.52 ± 0.10 (3)[d]	3.63 ± 0.76 (3)[d]

[a]Duration of perfusion was 2 hr.

[b]Numbers in parentheses indicate the number of perfusions done.

[c]$p < 0.1$ (vs control).

[d]$p < 0.05$.

TABLE II

Effect of Lipoproteins on [1-^{14}C] Acetate Incorporation into Cholesteryl Esters of Surfactant and Residual Fractions

Medium concentration	Rate of acetate incorporation into cholesteryl esters	
	Surfactant	Residual
	pmol/mg protein/hr ± SEM[a]	
Control (0)	0.23 ± 0.08 (5)[b]	1.82 ± 0.28 (5)
0.5 mM LDL cholesterol	0.27 ± 0.04 (4)	3.10 ± 0.34 (4)
2.5 mM LDL cholesterol	0.58 ± 0.26 (3)	2.46 ± 0.03 (3)
2.5 mM HDL cholesterol	0.16 ± 0.03 (3)	2.02 ± 0.06 (3)

[a]Duration of perfusion was 2 hr.

[b]Numbers in parentheses indicate the number of perfusions done.

TABLE III

Uptake of Lipoprotein [1,2-^{3}H] Cholesterol into Surfactant and Residual Fractions

Medium concentration	Rate of cholesterol uptake	
	Surfactant	Residual
	pmol/mg protein/hr ± SEM[a]	
1.0 mM LDL cholesterol	1.95 ± 0.14 (4)[b]	13.2 ± 1.7 (4)
1.0 mM HDL cholesterol	3.14 ± 1.26 (4)	10.9 ± 2.0 (4)
1.0 mM LDL/VLDL cholesterol[c]	0.94 ± 0.10	9.65 ± 2.52

[a]Duration of perfusion was 1 hr.

[b]Numbers in parentheses indicate the number of perfusions done.

[c]After Hass and Longmore (14).

1, it is shown that unlabeled LDL readily competed with ^{125}I-LDL for binding, whereas addition of unlabeled HDL resulted in no competition or possibly some increase in ^{125}I-LDL binding. Subsequent perfusion with medium containing 10 mg/ml heparin resulted in the release of bound ^{125}I-LDL from lung receptors (data not shown). Figure 2 shows the results of competitive binding studies using ^{125}I-HDL. Unlabeled medium HDL strongly competed with ^{125}I-HDL, whereas unlabeled LDL exhibited some competition only at very high concentrations. Thus, the lung appears to have receptors for the binding of both LDL and HDL. Experiments were also undertaken in which the uptake of ^{125}I-LDL and ^{125}I-HDL by the perfused lung was measured. As shown in Figure 3, ^{125}I-LDL were taken up by the lung at a nearly linear rate after a 5-10 min lag. In contrast, ^{125}I-HDL were taken up by the lung at a much reduced rate, similar to that for ^{125}I-LDL during the initial 10 min of perfusion. Calculations indicated that this difference could not be explained on the basis of ^{125}I-lipid uptake, but reflected differing rates of uptake of the apoprotein moieties.

DISCUSSION

The finding that lung cholesterol metabolism is under the regulatory control of serum lipoproteins is consistent with previous results which showed that endogenous cholesterol synthesis accounted for less than 1% of the total cholesterol of both the surfactant and residual fractions (14). That acetate incorporation into cholesterol of the surfactant and residual fractions was inhibited to a similar degree by both LDL (48% and 59%, respectively) and HDL (51% and 53%, respectively) is further indication that endogenous synthesis of surfactant cholesterol is subject to the same type of regulation as that of the residual fraction. Similarly, incorporation of ^{3}H-cholesterol from both LDL and HDL into the surfactant and residual fractions must result at least in part from uptake, rather than simple exchange, of cholesterol, since exchange would not be expected to result in inhibition of endogenous synthesis.

Lipoprotein inhibition of lung cholesterol synthesis, combined with the demonstration of lung lipoprotein receptors indicates that regulation of cholesterol metabolism in the lung parallels that reported for other tissues (13,26). That some competition between LDL and HDL for binding was observed at high lipoprotein concentrations likely results from the 5-10%

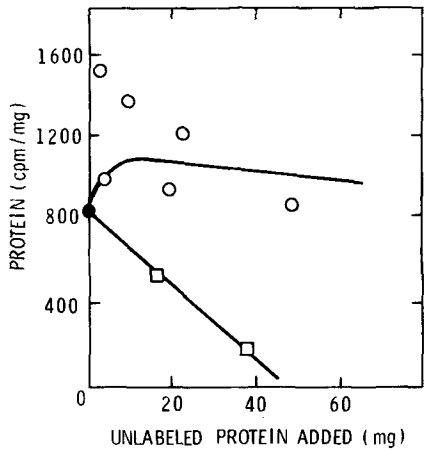

FIG. 1. Competitive binding of lipoproteins by perfused lung. Perfusion medium contained 6.5 mg ^{125}I-LDL plus varying amounts of unlabeled LDL (-□-), unlabeled HDL (-○-) or no competing lipoprotein (-●-). As described in Methods, lungs were perfused with ^{125}I-LDL for 5 min. Data points represent ^{125}I-LDL bound to lung following 5 min perfusion with unlabeled medium.

contamination of the HDL fraction by LDL. It is also possible that some common binding sites exist for both LDL and HDL, or that the much larger LDL molecules exert some steric hindrance in preventing HDL binding. Similar low level competition has been reported by Miller et al. (27) for human fibroblasts. The small increase in ^{125}I-LDL binding caused by addition of HDL (Fig. 1) is also similar to that seen in similar experiments using certain HDL fractions with human fibroblasts (28).

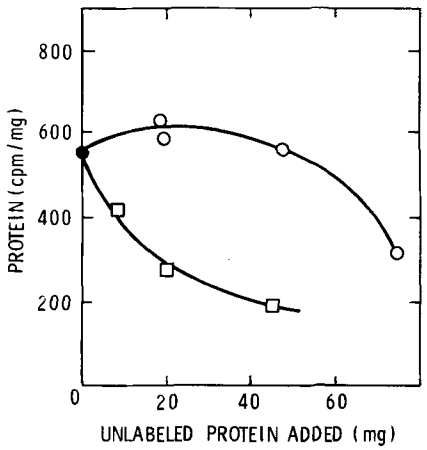

FIG. 2. Competitive binding of lipoproteins by perfused lung. Perfusion medium contained 6.5 mg ^{125}I-HDL plus varying amounts of unlabeled LDL (-○-), unlabeled HDL (-□-) or no competing lipoprotein (-●-). As described in Methods, lungs were perfused with ^{125}I-HDL for 5 min. Data points represent ^{125}I-HDL bound to lung following 5-min perfusion with unlabeled medium.

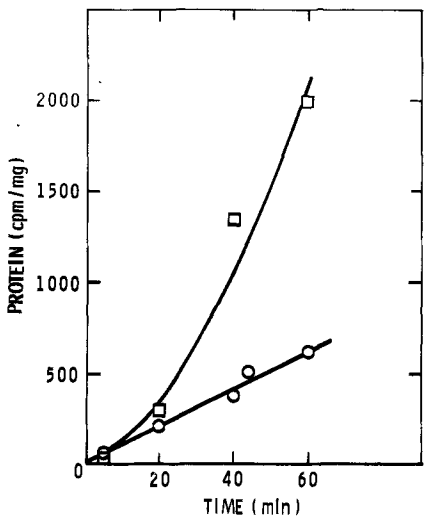

FIG. 3. Uptake of lipoproteins by perfused lung. Perfusion medium contained 25 mg/ml ^{125}I-LDL (-□-) or ^{125}I-HDL (-○-).

While the rate of uptake of lipoprotein cholesterol by the perfused lung is similar for both LDL and HDL, the rates of uptake of the protein portion of the molecule are significantly different. Calculation of the ratio of ^{125}I-labeled lipoproteins taken up during a 60-min perfusion to those which were bound yields a value of 535 for LDL and 13.4 for HDL. This would suggest that the LDL may be taken up by the lung as an intact particle, whereas HDL cholesterol is transferred without mandatory uptake of the apoprotein moiety. Similar findings have been reported for L cells and for human fibroblasts (29,30).

Pietra et al. (11) reported uptake by the perfused rat lung of ^{125}I-VLDL but not of ^{125}I-LDL. However, their methods entailed monitoring the disappearance of labeled lipoproteins from the perfusion medium. In this study, tissue radioactivity was measured. At no time did this tissue radioactivity represent more than 2.5% of the total medium radioactivity. Hence, the results of this study are not necessarily at odds with those reported by these authors.

Andersen and Dietschy (13) reported inhibition of endogenous cholesterol synthesis by rat lung slices after intravenous infusion of LDL to a serum cholesterol concentration of 4.4 mM. Infusion of LDL to a serum cholesterol concentration of only 1.3 mM caused a slight inhibition; similar results were reported for a comparable infusion of HDL. However, no parallel experiments with higher HDL concentrations were reported.

The dual regulation of lung cholesterol metabolism by LDL and HDL is similar to that reported by Andersen and Dietschy for the adrenal gland (13). It is interesting that the adrenal gland secretes a significant portion of its cholesterol in the form of corticosteroids. Similarly, the lung secretes up to 20% of its total cholesterol in the form of pulmonary surfactant. It is conceivable that tissues which metabolize cholesterol via multiple pathways require multiple forms of regulatory mechanisms to govern those pathways. Whether similar systems of dual regulation exist in other steroid-secreting tissues remains to be determined.

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Characterization of Triterpene Alcohols of Seed Oils from Some Species of Theaceae, Phytolaccaceae and Sapotaceae[1]

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ABSTRACT

Triterpene alcohol constituents of the unsaponifiable lipids separated from tea seed oil from *Thea sinensis* L. (Theaceae), camellia seed oil from *Camellia japonica* L. (Theaceae), pokeweed seed oil from *Phytolacca americana* L. (Phytolaccaceae) and shea butter from the seed kernels of *Butyrospermum parkii* (Sapotaceae) were studied. Among a number of triterpene alcohols present in these oils, 19 components were identified as cycloartenol, 24-methylenecycloartanol, parkeol, 24-methylene-24-dihydroparkeol, lanosterol, euphol, butyrospermol, tirucallol, tirucalla-7,24-dienol, dammaradienol, 24-methylenedammarenol, α-amyrin, β-amyrin, lupeol, germanicol, taraxasterol, ψ-taraxasterol, taraxerol and myricadiol. Tirucalla-7,24-dienol and butyrospermol are the predominant components of the 2 Theaceae and pokeweed seed oils. Shea butter, on the other hand, contains α-amyrin followed by butyrospermol and lupeol as the major triterpene constituents.

NOMENCLATURE

Cycloartenol = 9β,19-cyclo-5α-lanost-24-en-3β-ol (I), 24-methylenecycloartanol = 24-methylene-9β,19-cyclo-5α-lanostan-3β-ol (II), parkeol = 5α-lanosta-9(11),24-dien-3β-ol (III), 24-methylene-24-dihydroparkeol = 24-methylene-5α-lanost-9(11)-en-3β-ol (IV), lanosterol = 5α-lanosta-8,24-dien-3β-ol (V), euphol = 5α-eupha-8,24-dien-3β-ol (VI), butyrospermol = 5α-eupha-7,24-dien-3β-ol (VII), tirucallol = 5α-tirucalla-8,24-dien-3β-ol (VIII), tirucalla-7,24-dienol = 5α-tirucalla-7,24-dien-3β-ol (IX), dammaradienol = 5α-dammara-20,24-dien-3β-ol (X), 24-methylenedammarenol = 24-methylene-5α-dammar-20-en-3β-ol (XI), α-amyrin = 5α-urs-12-en-3β-ol (XII), β-amyrin = 5α-olean-12-en-3β-ol (XIII), lupeol = 5α-lup-20(29)-en-3β-ol (XIV), germanicol = 5α-olean-18-en-3β-ol (XV), taraxasterol = 5α-taraxast-20(29)-en-3β-ol (XVI), ψ-taraxasterol = 5α-taraxast-20-en-3β-ol (XVII), taraxerol = 5α-taraxer-14-en-3β-ol (XVIII), myricadiol = 5α-taraxer-14-ene-3β,28-diol (XIX), erythrodiol = 5α-olean-12-ene-3β,28-diol (XX).

INTRODUCTION

In a previous study on the triterpene alcohol constituents of Theaceae and some other seed oils, butyrospermol (VII), β-amyrin (XIII) and lupeol (XIV) from tea seed oil, and parkeol (II), VII, α-amyrin (XII) and XIV from shea butter, were isolated and identified (1). Moreover, continued studies on these oils resulted in our finding 3 new triterpene alcohols: 24-methylene-24-dihydroparkeol (IV) (2) and 24-methylenedammarenol (XI) (3) in shea butter and tirucalla-7,24-dienol (IX) in tea seed oil (4). However, the compositions of triterpene alcohol mixtures of these oils are much more complicated and a number of minor components remain to be identified. Therefore, the work reported here was undertaken to gain further information on the triterpene alcohol constituents of 2 Theaceae seed oils from tea (*Thea sinensis* L.) and camellia (*Camellia japonica* L.), pokeweed seed oil from *Phytolacca americana* L. (Phytolaccaceae) and shea butter from the seed kernels of *Butyrospermum parkii* (Sapotaceae).

EXPERIMENTAL

Crystallizations were performed in acetone/methanol. Melting points (mp) taken on a heat block were uncorrected. Infrared (IR) spectra were recorded in KBr on a Type IRA-2, IR spectrophotometer (Japan Spectroscopic Co., Tokyo). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained with a JNM-FX 100 instrument (Japan Electron Optics Laboratory Co., Tokyo) at 100 MHz in deuteriochloroform. Mass spectra (MS, 70 eV) of the isolated and unisolated compounds were taken on a Hitachi RMU-7M mass spectrometer with a direct inlet system (Hitachi Ltd., Tokyo) or on a Shimadzu LKB-9000 gas chromatograph-mass spectrometer (Shimadzu Seisakusho Ltd., Kyoto; 2% OV-17, 2 m x 3 mm id glass column). Gas liquid chromatography (GLC) was performed with a Shimadzu GC-4CM instrument equipped with a hydrogen flame ionization detector. Either Poly I-110 (column 270 C, injection 285 C, carrier gas N_2, 80 ml/min, split ratio 50:1, scavenge gas 80 ml/min)

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or OV-17 (column 260 C, injection 280 C, carrier gas N_2, 60 ml/min, split ratio 50:1, scavenge gas 80 ml/min) with SCOT glass capillary column (30 m x 0.3 mm id, Wako Pure Chemical Industries Ltd., Osaka) was used. Argentation (silver nitrate/silica gel, 1:4, w/w) preparative thin layer (0.5 mm thick) chromatography (TLC) was developed 4-6 times with methylene chloride/carbon tetrachloride (1:5, v/v). Extraction and saponification of seed oil and fractionation by TLC on silica gel of the unsaponifiable lipid using *n*-hexane/ethyl ether (7:3, v/v) as developer were performed similar to methods described previously (1). The triterpene alcohol (3β-monohydroxy triterpene) fraction was acetylated by acetic anhydride/pyridine and the resulting acetate fraction was further fractionated by argentation TLC. The resulting separate bands and fractions were numbered in the order of polarity, beginning with the least polar. Identification of the unisolated compounds was carried out by argentation TLC and GLC of the 2 columns in all cases and further by combined GC-MS in some cases, if necessary. The identity of each isolated compound was confirmed by comparison of its IR, ^{1}H-NMR and mass spectra and mp to those of an authentic specimen with the exceptions of ψ-taraxasterol (XVII) and erythrodiol (XX), the authentic specimens of which were unavailable.

Materials

Tea seed oil from Izome Oil Chemical Industries Co., Shizuoka, pokeweed seeds from Prof. T. Mitsuhashi, Tokyo Gakugei University, Tokyo, and shea butter from Fuji Oil Manufacturing Co., Osaka, were courteously supplied as gifts. Camellia seeds were collected in 1977 at a local camellia grove.

The following authentic specimens were used in this study: cycloartenol (I), 24-methylenecycloartanol (II), parkeol (III) and 24-methylene-24-dihydroparkeol (IV) (2); lanosterol (V) (5); euphol (VI) and tirucallol (VIII) (T. Itoh, T. Tamura and T. Matsumoto, unpublished results); butyrospermol (VII), α-amyrin (XII), β-amyrin (XIII) and lupeol (XIV) (1); tirucalla-7,24-dienol (IX) (4); and dammaradienol (X) and 24-methylenedammarenol (XI) (3) were prepared in this laboratory; and germanicol (XV) from Dr. W.-H. Hui, University of Hong Kong (Hong Kong) and Dr. A.G. González, Instituto de Productos Naturales Organicos del CSIC (IPNO), La Laguna (Spain); taraxasterol (XVI) and myricadiol (XIX) from Dr. W.-H. Hui; and taraxerol (XVIII) from Prof. T. Ohmoto, Toho University, Chiba, were generously supplied as gifts.

RESULTS

Triterpene Alcohols of Tea Seed Oil

Saponification of tea seed oil (100 g) gave unsaponifiable lipid (820 mg), which afforded a triterpene alcohol fraction (420 mg) upon TLC. A portion of the fraction (120 mg), upon acetylation, yielded the acetate fraction (128 mg), which was then separated into 6 major bands upon argentation TLC. Fraction 1 (9.4 mg) was a mixture of the acetates of α-amyrin (XII) and 2 unidentified components. Fraction 2 gave β-amyrin (XIII) acetate (7.6 mg), mp 243-245 C. Fraction 3 (6.8 mg) was composed of the acetates of germanicol (XV) and several unidentified components, which upon further argentation TLC afforded XV-acetate (2.5 mg), mp 279-281 C (lit. [6] mp 281-282 C). Hydrolysis of the acetate afforded free XV, mp 171-181 C (lit. [7] mp 181-182 C). Fraction 4 (7.4 mg) was a mixture of the acetates of XV and taraxerol (XVIII), which upon further argentation TLC afforded XVIII-acetate (2 mg), mp 286-288 C. Fraction 5 (52 mg) comprised the acetates of euphol (VI), butyrospermol (VII), tirucallol (VIII), tirucalla-7,24-dienol (IX), lupeol (XIV), ψ-taraxasterol (XVII) and 3 unidentified components. Fraction 6 (9.4 mg) upon further argentation TLC gave dammaradienol (X) acetate (3 mg) accompanied by a trace amount of 24-methylenedammarenol (XI) acetate, mp 153-154 C.

Triterpene Alcohols of Camellia Seed Oil

The oil (1080 g) extracted from the dried and ground cammellia seeds (1860 g) gave unsaponifiable lipid (3.6 g). A portion of the unsaponifiable lipid (1.9 g) upon TLC afforded a triterpene alcohol fraction (658 mg), and the acetate fraction (600 mg) derived from this was separated into 6 major bands upon argentation TLC. Fraction 1 (67 mg) was a mixture of the acetates of XII and XIII, and its further argentation TLC gave XIII-acetate (36 mg), mp 245-247 C. Fraction 2 (13 mg) comprised the acetates of VI, VII, VIII, IX, taraxasterol (XVI), XVII and XVIII. XVIII-Acetate was then isolated by further argentation TLC, mp 286-289 C. Fraction 4 (132 mg) was composed of the acetates of VII, IX, XIV and 2 unidentified components; and upon further argentation, TLC afforded XIV-acetate (15 mg), mp 209-213 C. Fraction 5 (151 mg) from the most polar band was a mixture of X-acetate and XI-acetate and further argentation TLC gave X-acetate (24 mg), mp 149-152 C.

Triterpene Alcohols of Pokeweed Seed Oil

The oil (150 g) extracted from the dried and

ground pokeweed seeds (1600 g) afforded unsaponifiable lipid (3.1 g). The lipid yielded a triterpene alcohol fraction (230 mg) and 4-desmethylsterol fraction (520 mg) on TLC. The triterpene alcohol fraction, after acetylation (240 mg), was separated into 7 major bands upon argentation TLC. Fraction 1 (12 mg) was a mixture of the acetates of XII and XIII. Fraction 2 (8 mg) upon further argentation TLC gave XVIII-acetate (2 mg), mp 251-252 C. Fraction 3 (7 mg) was composed of cycloartenol (I) acetate and VI-acetate. Repeated argentation TLC of the fraction gave I-acetate (2 mg), mp 120-123 C. Fraction 4 (13 mg) was a mixture of the acetates of VIII, IX, XIV and XVII. Fraction 5 (60 mg) was a mixture of the acetates of VII, IX and 24-methylenecycloartanol (II). Fraction 6 (22 mg) comprised the acetates of parkeol (III), 24-methylene-24-dihydroparkeol (IV) and 2 unidentified components, and further argentation TLC afforded IV-acetate (2 mg), mp 156.5-157.5 C. Fraction 7 (30 mg) upon further argentation TLC yielded X-acetate (3 mg) accompanied by a trace amount of XI-acetate, mp 151.5-152.5 C.

Crystallization of the 4-desmethylsterol fraction (already described) after acetylation (530 mg) gave a filtrate (250 mg). The fraction (60 mg) from the most polar band on argentation TLC of the filtrate gave myricadiol (XIX) diacetate (36 mg), mp 261-263 C. XIX-Diacetate (20 mg) in acetic acid (5 ml) was heated for 10 min on a hot plate in the presence of conc. hydrochloric acid (0.5 ml). Usual work-up of the reaction mixture followed by purification on TLC yielded erythrodiol (XX) diacetate (16 mg), mp 191-194 C (lit. [7] mp 185-186 C). The MS of the diacetate agreed well with that reported for XX-diacetate (8). Hydrolysis of the acetate gave free XX, mp 236-238 C (lit. [7] mp 239-240 C).

Triterpene Alcohols of Shea Butter

Shea butter (100 g) yielded unsaponifiable lipid (4.2 g). The triterpene alcohol fraction (1.13 g) separated from the unsaponifiable lipid (1.96 g) by TLC was acetylated and the acetate fraction (1.04 g) was first subjected to crystallization. The crystallization portion (400 mg) was a mixture consisting mainly of XII and XIV-acetates. The filtrate (600 mg) upon argentation TLC was separated into 6 major bands. Fraction 1 (49 mg) was a mixture of XII and XIII-acetates. Fraction 2 (14 mg) upon repeated argentation TLC afforded XV-acetate (3 mg), mp 279-281 C. Fraction 3 (53 mg) was further fractionated into 4 fractions by argentation TLC. Fraction 3-1 was a mixture of the acetates of lanosterol (V) and XVII, which upon further crystallization gave XVII-acetate (2 mg), mp 240.5-243 C (lit. [6] mp 243-244 C, [9] mp 238-240). The MS of the acetate was identical to that reported for XVII-acetate (9). Fraction 3-2 was a mixture of the acetates of V, VI, XVI, XVII and XVIII. Fraction 3-3 comprised the acetates of V, XIV and XVI. Fraction 3-4 was a mixture of the acetates of VII, IX, XIV and several other components. Fraction 4 (305 mg) consisted of VII and XIV-acetates. The acetates of III-IV and X-XI were the major constituents of fraction 5 (25 mg) and fraction 6 (20 mg), respectively.

DISCUSSION

The combined use of GLC on 2 SCOT glass capillary columns, the more polar Poly I-110 column and the less polar OV-17 column, and argentation TLC in this study was very effective in detecting and identifying a number of minor components, most of which were undetermined previously (1), present in the triterpene alcohol mixtures of the seed oils investigated. Table I shows the approximate Rc-values (Rf-values relative to cholesterol acetate) on argentation TLC and the methylene indices on the 2 columns in GLC of the acetates of 20 triterpene alcohols.

Nineteen triterpene alcohols were identified in this study. Approximate quantitative compositions of the triterpene alcohol fractions from the 4 oils shown in Table II were determined mainly based on the GLC data on Poly I-110 column. The triterpene alcohol mixtures of the seed oils from 2 Theaceae and pokeweed are found alike in their compositions, characterized by the predominance of the 2 Δ^7-tirucallane/euphane compounds, tirucalla-7,24-dienol (IX) and butyrospermol (VII). Shea butter, on the other hand, contains α-amyrin (XII) as the predominant component. IX was first isolated as a uniform component from the triterpene fraction of tea seed oil (1) and its structure was recently determined (4). Since the chromatographic mobility on argentation TLC and on OV-17 column in GLC of this compound (IX) is closely approximated to that of lupeol (XIV) (see Table I), a common pentacyclic triterpene, the separation and quantification of the compound (IX) by means of GLC on the packed OV-17 column alone were unsuccessful in the previous study (1). However, a Poly I-110 SCOT glass capillary column in GLC, the 2 components IX and XIV were fully resolved.

The vegetable oils investigated here are distinguished by their triterpene composition from a number of vegetable oils which contain

TABLE I

Approximate Rc-Values on Argentation TLC and Methylene Indices on GLC of Triterpene Acetates

	Triterpene acetate	Approximate Rc-value[a]	Methylene indices	
			Poly I-110	OV-17
I	Cycloartenol	0.68	37.78	36.85
II	24-Methylenecycloartanol	0.38	38.12	37.22
III	Parkeol	0.20	37.30	36.65
IV	24-Methylene-24-dihydroparkeol	0.13	37.76	37.07
V	Lanosterol	0.83	36.85	36.20
VI	Euphol	0.84	36.10	35.43
VII	Butyrospermol	0.31	37.13	36.46
VIII	Tirucallol	0.64	36.55	35.98
IX	Tirucalla-7,24-dienol	0.41	37.61	36.98
X	Dammaradienol	0.11	36.90	36.38
XI	24-Methylenedammarenol	0.09	37.25	36.65
XII	α-Amyrin	1.49	37.96	36.78
XIII	β-Amyrin	1.46	37.38	36.38
XIV	Lupeol	0.42	37.83	36.97
XV	Germanicol	1.03	37.38	36.38
XVI	Taraxasterol	0.60	39.11	37.92
XVII	ψ-Taraxasterol	0.64	39.01	37.82
XVIII	Taraxerol	0.76	37.28	36.16
XIX	Myricadiol	0.34	40.47	39.95
XX	Erythrodiol	---	40.47	40.10

[a]Approximate relative mobility to cholesterol acetate (Rf = 0.46) on argentation TLC (5 developments).

TABLE II

Approximate Composition (%) of Triterpene Alcohol Fractions from the Unsaponifiable Lipids of Theaceae and Some Other Vegetable Seed Oils[a]

Triterpene alcohol	Percent composition			
	Tea	Camellia	Pokeweed[b]	Shea
Euphol (VI)	0.4	0.4	0.3	0.3
Tirucallol (VIII)	Trace	0.4	Trace	---
Dammaradienol (X)	7.9	7.4	3.2	0.5
Lanosterol (V)	---	---	---	
Butyrospermol (VII)	27.7	19.4	27.4	12.3
Taraxerol (XVIII)	4.5[c]	2.8[c]	3.9	0.3[c]
24-Methylenedammarenol (XI)				
Parkeol (III)	---	---		1.5
β-Amyrin (XIII)	15.3[d]	27.3[d]	2.4	7.1[d]
Germanicol (XV)			---	
Tirucalla-7,24-dienol (IX)	31.4	28.1		0.8
24-Methylene-24-dihydroparkeol (IV)	---	---	40.3[e]	0.4
Cycloartenol (I)	---	---		---
Lupeol (XIV)	5.0	6.3	4.7	17.0
α-Amyrin (XII)	Trace	0.5	0.7	54.6
24-Methylenecycloartanol (II)	---	---	11.3	---
ψ-Taraxasterol (XVII)	0.1	1.6	1.0	2.6
Taraxasterol (XVI)	---	Trace	---	1.0
Others, unidentified	7.7	5.8	4.8	1.6

[a]Roughly determined values based mainly on the GLC data on Poly I-110 column.
[b]Myricadiol (XIX) also is contained.
[c]XVIII >> XI.
[d]XIII > XV.
[e]IX > I, IV.

cycloartane (9β,19-cyclolanostane) triterpenes, cycloartenol (I) and 24-methylenecycloartanol (II), as the major triterpene constituents (5,10-13). These 2 cycloartane compounds were detected here only in pokeweed seed oil as the minor components accompanied with their $\Delta^{9(11)}$-isomers, parkeol (III) and 24-methylene-24-dihydroparkeol (IV). In shea butter, minute amounts of the 2 $\Delta^{9(11)}$-compounds and lanosterol (V), a Δ^{8}-isomer of I were present, but neither of the 2 cycloartane triterpenes could be identified.

Recently, a triterpene alcohol found in shea butter was reported to be germanicol (XV) (14), but an inspection of the GLC data cited therein seems to indicate that it might be ψ-taraxasterol (XVII) or taraxasterol (XVIII) rather than XV.

A triterpene diol, isolated from the 4-desmethylsterol fraction of pokeweed seeds, was identified as myricadiol (XIX). The identity of XIX was confirmed further by its acid isomerization to erythrodiol (XX) (15,16). The triterpene (XIX) was first isolated from the bark of *Myrica gale* L. (Myricaceae) and its structure was indicated as taraxer-14-en-3β,28-diol (15). The compound was later detected in the bark of *Luvunga scandens* Ham. (Rutaceae) (16) and the stems of *Lithocarpus cornea* (Lour.) Rhed. (Fagaceae) (17). The seeds of pokeweed were recently shown to contain also 3-acetylaleuritolic acid (3β-acetyltaraxer-14-en-28-oic acid), the corresponding mono-acid of XIX (18).

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Utilization of Polyunsaturated Fatty Acids by Human Diploid Cells Aging in vitro

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ABSTRACT

Cultures of human diploid cell strain IMR-90 were supplemented with γ-linolenic acid, 18:3ω6, by constant infusion over 72 hr. Cell growth was twice that observed when the same amount of fatty acid was supplied as a single dose at the start of a 72-hr incubation. Using the infusion method, growth of cells receiving monoenoic or polyenoic fatty acids was compared. The age of these cells in vitro was measured in terms of the culture mean population doubling level (PDL). Population doubling level refers to the mean number of doublings elapsed since establishment of a primary culture. At PDL from 24-53, the growth of cells from cultures supplemented with oleic acid was similar to that of noninfused cultures. Gamma linolenic acid, 18:3ω6, and to greater extent arachidonic acid, 20:4ω6, however, caused suppression of cell multiplication at PDL $\leqslant$ 32, but not at PDL $\geqslant$ 44. The polyunsaturated fatty acid (PUFA) levels in cell phospholipids were reduced by exogenous oleic acid to half that of nonsupplemented cells at all PDL tested. Conversely, the PUFA levels in phospholipids were elevated by a factor of 1.6 at all PDL when cultures were infused with 18:3ω6. Triglyceride levels at the end of 72 hr were similar, but much higher than the controls, regardless of the fatty acid supplied. Growth inhibition, modification of phospholipid acyl group content and triglyceride levels were not appreciably affected when the amount of monoenoic or polyenoic fatty acid infused into the cultures was doubled. The elongation of 18:3, as well as the distribution of 18:3 and its elongation products, between triglyceride and phospholipid, was dependent on whether the 18:3 was of the ω3 or ω6 family.

INTRODUCTION

Only recently have there been attempts to modify the phospholipid acyl group composition of normal diploid cells having a finite life span in vitro (1,2). The growth of these cells is apparently more sensitive to the addition of exogenous fatty acid salts than is that of transformed cell lines (1, and Lynch, unpublished observations). Although the reason for this difference is unclear, the addition of fatty acid salts as a single dose to transformed or nontransformed cells results in the rapid formation of cytoplasmic triacylglycerol (1-4). This accumulation may be great enough to disrupt cells or, at the very least, cause shape changes (3). Although this may be of little consequence to transformed cells, the shape of nontransformed cells may play an important role in regulating their passage through the cell cycle (4). In earlier studies, to reduce triglyceride accumulation in strain L-fibroblasts, a transformed cell line, the fatty acid salt solution was infused into suspension cultures over a 48-hr period (5). This resulted in modification of the phosphoglyceride fatty acid composition to at least as great an extent as that observed after the administration of the same amount of fatty acid as a single dose at the start of the 48-hr period while maintaining cellular triglyceride at much lower levels.

The purpose of this study was 3-fold: (a) to determine whether the previously reported inhibition of growth of normal human diploid lung fibroblasts (IMR-90) by oleic acid (1) or polyunsaturated fatty acids (PUFA) could be eliminated or reduced by infusing fatty acids into the culture; (b) to define conditions which maximally depress or enhance phosphoglyceride PUFA composition of diploid cells while minimizing triglyceride accumulation; and (c) to determine whether, as has been shown for endogenous biosynthesis of fatty acid (6), the age in vitro of the culture affects the use of exogenous fatty acids by IMR-90 cells.

MATERIALS AND METHODS

Cell Culture

Human female fetal lung fibroblasts, strain IMR-90, at population doubling level 10 (PDL 10) were purchased from the Human Aging Cell Repository of the Institute for Medical Research (Camden, NJ). Population doubling level refers to the mean number of cell doublings elapsed since establishment of a primary culture. This cell strain has been characterized thoroughly and is similar to the strain WI-38 employed in other studies of aging in vitro (7). In this laboratory, these cultures normally cease multiplying after 55 population doublings.

Stock cultures of cells were routinely propagated as monolayers in plastic tissue culture flasks with a 75-cm^2 growth area.

Monolayers were covered with 12 ml of Eagles Minimum Essential Medium (MEM) (Grand Island Biologicals, Inc., Grand Island, NY) supplemented to a level of 10% with nonheat-inactivated fetal calf serum (Grand Island Biologicals, Inc., Grand Island, NY). For buffering, the medium contained 5 mM N-2 hydroxethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES), pH 7.3. All stock cultures were antibiotic-free and were maintained at 36.5 C in a humidified atmosphere of 5% CO_2 (New Brunswick CO-20 Incubator, New Brunswick, NJ). Cultures were periodically monitored for mycoplasma contamination (8).

When confluent, cells were detached using a releasing medium of 0.005% trypsin TRL (Worthington Biochemical Corp., Freehold, NJ) in an isotonic solution containing 0.5 mM NaEDTA, buffered with 5 mM HEPES. Sodium bicarbonate was added to the trypsin solution to a concentration of 7 mM just prior to use; the final pH was 7.2-7.3. Briefly, the growth medium was replaced with trypsin solution (4.5 ml) and the culture was incubated for 5 min at 37 C. The action of the trypsin was lessened by addition of fresh medium (8.5 ml) and the cells then detached by gentle tapping. Clumps of cells were disrupted by passing the cell suspension up and down 3 or 4 times using a 10-ml pipet with a narrow orifice. The suspension (3 ml) was transferred to fresh medium (9 ml, 1:4 split ratio) and 0.5 ml diluted with saline to obtain a cell count using a Coulter counter model ZBI (Coulter Electronic, Hialeah, FL). The remaining cell suspension was used to seed flasks (25 cm^2 growth area) for experimental purposes. Stock cultures at PDL < 40 attained confluency within 7 days of a 1:4 split, whereas those at higher passage levels required 10-14 days. Between subculture, the medium was changed every 3 or 4 days. The yield from confluent cultures under these conditions was between 20-30 x 10^6 cells/flask.

Preparation of Fatty Acid Salt Solution

Oleic (18:1ω9), α-linolenic (18:3ω3), γ-linolenic (18:3ω6) and arachidonic (20:4ω6) acids were purchased from Applied Science, Inc. (State College, PA) and used without further purification. Based on gas chromatography (GC) of their methyl esters, the purity of these fatty acids was judged to be at least 99%. (All fatty acids are denoted by number of carbon:number of double bonds; the figure following the ω symbol indicates the first C-atom, starting from the CH_3 end of the chain, at which a double bond is encountered). Sealed ampoules of these fatty acids were opened in a nitrogen atmosphere and the contents quickly diluted with sufficient heptane to yield a final concentration of ca. 50 mg/ml. An accurately measured volume was dried under N_2 in a clean tared vial to determine the fatty acid concentration more precisely. The stock solutions were then stored in liquid N_2. Before use, the required amount of stock solution was washed routinely with 2 vol of a mixture containing methanol/H_2O (3:1, v/v) to remove any trace quantities of lipid hydroperoxides which might be present (9). The washed heptane phase was then dried under N_2 and the fatty acid solution immediately dissolved in 0.12% NaOH; the salt was frozen in liquid N_2.

Preparation of MEM-fatty Acid Salt-albumin Complex

Fatty acid-free bovine serum albumin purchased from Miles Laboratory, Inc. (Elkhart, IN) was dissolved in Eagles MEM. The mixture was warmed rapidly to 56 C, the fatty acid salt added while stirring and the resulting solution chilled quickly to 4 C in an ice bath (6). Amounts of albumin and fatty acid were adjusted so that the molar ratio of fatty acid: albumin was equal to 4:1. These solutions were either stored overnight in liquid N_2 or used immediately. Just prior to use, the solution was passed through a sterile 0.22-μm millipore filter (Millipore Corp., Bedford, MA). Samples of the infusate were tested for malonaldehyde, a product of lipid peroxidation, at the start and finish of the 72-hr incubation period (10). The amounts measured were at the lower limit of detection with the method used. Furthermore, the amount of PUFA recovered by extraction from the infusate was determined by GC after methylation in the presence of heptadecanoic acid and agreed, within experimental error, with the expected value.

Addition of Fatty Acid-albumin Solution to Monolayer Culture

For experiments, cell suspensions from freshly trypsinized cultures were diluted with fresh medium and seeded into plastic flasks (25 μm^2 growth area), at an initial density of ca. 3.2 x $10^4/cm^2$ and left undisturbed for 3 days. At this time, when cells had increased to 4.8 x $10^4/cm^2$ and were in log phase, the medium was removed and fresh medium (2.0 ml) containing penicillin (10,000 units) and streptomycin (.013 g) was added. The MEM solution of fatty acid-albumin complex was then added to the system by one of 2 methods: (a) the total amount of fatty acid-albumin in a vol of 3.0 ml was added as a single dose at the start of a 72-hr incubation; or (b)

the same vol of complex was infused into the culture at a constant rate throughout the 72 hr. This was done using a constant infusion pump (Sage Instruments, Orion Research, Inc., Cambridge, MA) fitted with a 5.0-ml syringe which was connected by No. 22 teflon tubing to the experimental flask. Control cultures received the same vol of additional medium with the appropriate amount of albumin, but without fatty acid. All experimental flasks were placed on a platform rocker (Bellco Glass, Inc., Vineland, NJ) in the CO_2 incubator and gently rocked at 8 cycles/min for the duration of the experiment.

Harvesting Procedures

At the end of the infusion period, cells were trypsinized and shaken free of the plastic surface. The osmolarity of suspending medium was increased to 375 mOsm/ℓ by the addition of harvesting medium made hyperosmolar with sucrose. A hyperosmolar solution has been shown to increase the efficiency of recovery when small numbers of cells are harvested by centrifuging cell suspensions (11,12). The total vol of the suspension was adjusted to 7.0 ml and a 0.5-ml aliquot taken for cell count. The remainder was centrifuged for 5 min at 600 x g at 4 C; the pellet was washed once with trypsin-free releasing medium with an adjusted osmolarity of 375mOsm/ℓ and the cells were sedimented a second time.

Extraction and Separation of Lipids

A 25-μg aliquot of triheptadecanoin was added to the washed pellet, which was then extracted 3 times with $CHCl_3$/MeOH (2:1, v/v) and washed as described previously (13,14). After removing one-fourth of the lipid extract for total lipid phosphorous determination (15), the remainder was dried in a stream of N_2 and concentrated for thin layer chromatography (TLC).

Lipid samples were applied to a thin layer plate coated with a 0.4-mm-thick layer of silica gel impregnated with 0.11% ammonium sulfate (16). The solvent system, hexane/diethyl ether/acetic acid (60:39:1, v/v) separated the total lipid into the following classes in order of increasing R_f: (a) phospholipid; (b) sterol; (c) diglyceride; (d) fatty acid; (e) triglyceride; and (f) sterol esters. After development, the various lipid classes were visualized under ultraviolet (UV) illumination after spraying the plate with 0.1% aqueous solution of 8-anilino-1-napthalene sulfonic acid (ANS). Gel zones containing phospholipid and triglyceride were scraped into 15-ml screw-capped tubes and methylated in a solution of methanol/benzene (60:40, v/v) 1 N in NaOH (17). The resulting methyl esters were extracted into heptane, concentrated in a vol of 5-7 μl and an aliquot of 0.5-1.0 μl injected onto the gas chromatograph column.

Methyl esters of fatty acids were separated on a 6-ft glass column packed with 10% SP-2330-PS on 100/120 Chromosorb A AW (Supelco, Bellefonte, PA) mounted in a Varian Series 2440 gas chromatograph (Varian Associates, Waltham, MA). Areas under each peak were determined electronically by a Model 3380A electronic integrator (Hewlett Packard, Corvallis, OR). With the exception of 22:3ω6 and 20:4ω3, fatty acid methyl esters were identified by comparing their retention times with those of standards available commercially (Supelco). The 22:3ω6 and 20:4ω3 acids were only tentatively identified by comparing their retention times with those of either 18:3ω6 and 20:3ω6, or 20:3ω3 and 20:5ω3, respectively. Triglyceride levels were estimated by comparing the area under the methyl heptadecanoate peak with the sum of areas from the sample.

RESULTS

Cells which received the entire quantity of fatty acid at the start of an experiment (single-dose method) were covered by 5 ml of medium throughout the 72-hr incubation period. Those from cultures receiving fatty acid by infusion over 72 hr (infusion method), however, were initially covered by only 2.0 ml; the remaining 3.0 ml was added slowly during the 72-hr infusion period. Control cells receiving MEM-albumin under both sets of conditions grew to the same extent and had similar lipid compositions. All control cultures in the following experiments were therefore incubated without added fatty acid in a 5 ml vol of medium for the duration of the experiment. This allowed the limited number of spaces for syringes on the infusion pumps to be used for infusion of experimental cultures with fatty acids.

When added as a single dose at the start of a 72-hr incubation, 0.4μmol of 18:3ω6 reduced growth to 54% of control cultures (Table I). Infusing the same quantity of fatty acid into parallel cultures allowed cell growth to continue at 77% of control values. Using either method, the PUFA content in phospholipid and the amount of triglyceride/cell were elevated to a similar extent. As in the case of transformed cells (18), most of the increase in PUFA was at the expense of the monoenoic fatty acid fraction. The relatively high percentage of PUFA in the phospholipid of control cells most likely resulted from use of serum lipids (19). In

an effort to reduce this high PUFA background, fetal calf serum was delipidized as previously described (13,20) and replaced whole fetal calf serum in the medium. After 72 hr of incubation under these conditions, the percentage of PUFA in the phospholipid was reduced by 26%; however, culture growth was depressed by 50%. In all subsequent experiments, whole fetal calf serum was added to the culture medium.

Despite the use of the infusion method to reduce negative effects of fatty acid salts on culture multiplication, the growth of cells receiving PUFA was less than that of control cells or cells receiving oleic acid. Moreover, this residual effect on culture growth was apparent only at low PDL (Table II). At either high or low PDL, cells receiving 18:1 multiplied at the same rate as those from control cultures. The growth of cells from cultures infused with 20:4ω6, though, was markedly decreased when cells were at PDL $\leq$ 32 but was not reduced at PDL $\geq$ 44. The magnitude of the growth effect at low PDL was the same whether cells received 0.4 or 0.8 μmol of fatty acid. Similar, but less striking, effects were noted when 18:3ω6 was supplied.

TABLE I

Growth, Phospholipid Acyl Group Composition and Triglyceride Content of IMR-90 Cells[a] Supplemented with γ-Linolenic Acid by Two Methods[b]

Method of addition[c]	Amount of fatty acid (μmol)	Culture growth[d]	Phospholipid acyl groups (wt %)			Triglyceride (μg/10^6 cells)
			Saturated	Monoenoic	Polyenoic	
Control	---	3.34	30.1 ± 0.6	34.7 ± 1.0	35.2 ± 1.6	1.7 ± 0.5
Single dose	0.4	1.89	26.5 ± 4.2	17.9 ± 1.9	55.8 ± 6.4	10.2 ± 2.4
Infusion	0.4	2.57	28.1 ± 6.0	22.7 ± 3.8	49.2 ± 8.5	14.0 ± 0.2

[a]Cells had completed 32 population doublings and were in log phase at the start of the experiment.

[b]All data represent the mean ± SD of 3 separate experiments.

[c]Cultures received 0.4 μmol of 18:3ω6 either as a single dose at the beginning of a 72-hr incubation period or by infusion throughout that time.

[d]At the start of supplementation, cell numbers/flask = 1.26 x 10^6. The number of cells harvested after 72 hr ÷ the starting number = culture growth.

TABLE II

Growth Inhibition of IMR-90 Cells by Polyunsaturated Fatty Acids As a Function of Population Doubling Level[a]

Population doubling level	Amount of fatty acid (μmol[b])	Initial cell number[c] (cells x 10^{-6})	Increase in cell number of supplemented culture as a fraction of control cultures: Fatty acid supplied		
			18:1ω9	18:3ω6	20:4ω6
24	0.8	0.84	---	0.70[d]	0.55[e]
44	0.8	1.35	---	1.07[d]	0.98[e]
28	0.8	1.36	0.81[e]	---	0.50[e]
52	0.8	1.44	0.95[e]	---	1.09[e]
32	0.4	1.23	---	0.77[f]	0.62[f]
53	0.4	1.13	1.15[e]	1.18[e]	1.32[e]

[a]Population doubling level (PDL) is a measure of the lifespan of diploid cells in culture. In this laboratory, cells cease dividing and cell death is apparent after 52-55 population doublings in vitro.

[b]All fatty acids were supplied over a 72-hr period by infusion as described in Materials and Methods.

[c]This is the cell number 3 days after seeding the flasks, a time when the cells are in log phase.

[d]Results from a single experiment.

[e]Mean from 2 separate experiments.

[f]Mean from 3 separate experiments.

Four different PUFA were infused into log phase cultures of IMR-90 cells at an intermediate PDL and the resulting modification of phospholipid fatty acid compositions was compared with that produced when cells were incubated with 18:1 (Table III). In contrast to the results of a recent study (2) in which no change in phospholipid acyl group composition was reported after 18:1 supplementation, exogenous 18:1 suppressed phospholipid PUFA content of IMR-90 cells to a level half that observed in nonsupplemented control cells, whereas the monoenoic fatty acid content increased by a factor of 1.8. The mass of the triglyceride fraction increased above control values by a factor of 4.5 with its fatty acid composition reflecting closely that of the phospholipid (Table IV). Analysis of the lipid extract from fetal calf serum revealed the presence of 280 μg/ml of fatty acid, nearly all of which was in esterified form. The low level of triglyceride in control cells suggests, however, that this fatty acid is used slowly by the cells and does not affect the results obtained after fatty acid supplementation. The ω3 family of acids observed in the cells are present in the serum. No difference in fatty acid composition or triglyceride content was observed when cells were grown in the presence or absence of albumin.

Both the α and γ isomers of 18:3 were incorporated into cell glycerolipids (Tables III and IV); their fate within the cell, however, differed in 2 respects: (a) large increases in 20:3ω6 were observed in phospholipid triglyceride fractions of cells receiving 18:3ω6. In contrast, most of the 18:3ω3 was incorporated into cell lipids unchanged; (b) the distribution of 20:4 between phospholipid and triglyceride was dependent on whether it was of the ω3 or ω6 family, the ω6 being preferentially incorporated into phospholipid whereas the ω3 was sequestered primarily in the triglyceride fraction. No increases in the more highly polyunsaturated fatty acid 22:4 were observed in either the phospholipid or triglyceride frac-

TABLE III

Concentration and Acyl Group Composition of Phospholipids from IMR-90 Cells[a] Supplemented with Monenoic or Polyenoic Fatty Acids[b]

Phospholipid fatty acids	Phospholipid fatty acid composition (wt %)				
	Fatty acid infused[c]				
	0[d]	18:1	18:3ω6	18:3ω3	20:4ω6
Classes					
Saturated	28.8	24.2 ± 0.1	31.5 ± 0.3	32.5 ± 2.0	35.8 ± 0.8
Monoenoic	33.0	57.0 ± 1.3	19.0 ± 0.6	22.3 ± 2.0	23.4 ± 0.2
Polyenoic	38.2	18.9 ± 1.3	49.6 ± 0.9	45.3 ± 3.8	40.8 ± 0.6
Individual acids					
16:0	9.2	11.6 ± 0.4	15.2 ± 0.4	13.1 ± 2.9	18.4 ± 0.8
16:1	1.7	2.2 ± 0.2	2.0 ± 0.0	3.6 ± 2.4	3.2 ± 0.2
18:0	19.6	12.3 ± 0.1	17.1 ± 0.4	19.4 ± 1.0	17.4 ± 0.1
18:1	31.3	54.8 ± 1.1	17.0 ± 0.6	19.0 ± 0.3	20.2 ± 0.4
18:2	3.4	2.4 ± 0.3	2.9 ± 0.6	3.7 ± 0.4	3.6 ± 0.2
18:3ω6	0.6	tr[e]	8.6 ± 0.1	---	tr
20:3ω6	3.1	1.0 ± 0.2	17.2 ± 0.2	1.7 ± 0.1	2.0 ± 0.3
20:4ω6[f]	15.6	7.4 ± 0.2	10.0 ± 1.2	11.4 ± 1.2	21.3 ± 0.4
20:5ω3	---	---	---	3.9 ± 0.5	---
22:3ω6	---	---	2.2 ± 0.1	tr	---
22:4ω6	2.5	2.0 ± 0.2	2.9 ± 0.0	1.6 ± 0.2	10.2 ± 0.2
18:3ω3	---	---	---	13.3 ± 0.2	---
20:4ω3	---	---	---	2.3 ± 0.4	---
22:5ω3	5.2	2.8 ± 0.3	3.0 ± 0.1	4.3 ± 0.9	2.2 ± 0.1
22:6ω3	7.2	3.6 ± 0.5	3.0 ± 0.2	3.1 ± 0.7	1.1 ± 0.3
μg Lipid P per 10^6 cells	0.7	1.1 ± 0.0	1.1 ± 0.2	1.0 ± 0.1	1.4 ± 0.0

[a]At the start of infusion, cells/flask = 1.2 X 10^6; PDL = 32.

[b]Unless noted otherwise, all values are mean ± SD of 3 separate experiments.

[c]A total of 0.4 μmol of each fatty acid was infused into cultures over 72 hr.

[d]Values are mean of 2 experiments.

[e]tr = trace.

[f]20:4ω6 and 20:3ω3 have identical retention times on the packing material used.

tions. Exogenous arachidonic acid also increased the PUFA content of phospholipid, but to a smaller extent than that observed when either of the 18:3 isomers was used. Most of the increase in PUFA was in the 22:4 fraction; the 20:4 levels in phospholipid did not exceed 21% despite the presence of a triglyceride pool 8 times that of control cells with a 20:4ω6 content of 37%. Regardless of whether cells had completed 45% (PDL 24) or 95% (PDL 53) of their lifespan in vitro, the effects of fatty acid supplementation on the acyl group class distribution was similar in both the phospholipid and triglyceride fraction (Table V). Although not statistically significant, there was a trend toward a small increase in lipid phosphorous per 10^6 cells during supplementation with fatty acid. This may, in part, be related to a decrease in cell multiplication and an increase in cell size.

Further increases in the amount of PUFA delivered to cultures had little effect on glycerolipid acyl group composition. Doubling the amount of exogenous 18:1 increased the monoenoic acid content only slightly and caused no further suppression of the PUFA content in phospholipid (Table V). At the lower dose, 0.4 μmol of 18:1, the increase in 18:1 was compensated for by a decrease in PUFA with no change in saturated fatty acid content of the phospholipid, whereas at higher levels (0.8 μmol), both the saturated and PUFA fraction decreased in the phospholipid. Although there was no large increase in the amount of triglyceride per 10^6 cells at the higher level, the 18:1 content of that lipid class was elevated. There was little dose-dependent increase in phospholipid PUFA noted when the amount of exogenous PUFA was increased from 0.4 to 0.8 μmol, and as was reported in an earlier study with skin fibroblasts (2), arachidonic acid was least effective in eliciting a change in glycerolipid PUFA content. Doubling the quantity of 20:4 infused into the cultures caused the smallest change in the PUFA content of phospholipid despite the presence of

TABLE IV

Concentration and Acyl Group Composition of Triglyceride from IMR-90 Cells[a] Supplemented with Monoenoic or Polyenoic Fatty Acids[b]

Triglyceride fatty acids	Triglyceride fatty acid composition (wt %)				
	Fatty acid infused[c]				
	0[d]	18:1	18:3ω6	18:3ω3	20:4ω6
Classes					
Saturated	67.4	25.2 ± 3.8	17.0 ± 0.5	23.3 ± 5.6	21.8 ± 1.1
Monoenoic	7.9	55.0 ± 2.8	5.2 ± 0.7	9.9 ± 1.0	8.6 ± 2.8
Polyenoic	24.6	19.8 ± 6.6	80.6 ± 2.8	66.8 ± 4.9	69.6 ± 3.9
Individual acids					
16:0	35.2	13.6 ± 0.4	8.8 ± 1.5	14.6 ± 5.6	12.2 ± 2.0
16:1	tr[e]	1.4 ± 0.6	tr	tr	1.7 ± 1.3
18:0	32.2	11.6 ± 3.4	8.2 ± 1.0	8.5 ± 0.3	9.8 ± 1.8
18:1	7.9	54.2 ± 4.0	5.2 ± 0.7	9.9 ± 1.0	7.4 ± 0.9
18:2	2.4	2.2 ± 0.3	1.8 ± 0.5	4.2 ± 0.6	2.6 ± 0.3
18:3ω6	14.4	2.0 ± 1.5	16.3 ± 2.8	tr	3.9 ± 1.0
20:3ω6	5.9	6.4 ± 3.8	38.8 ± 1.6	2.0 ± 0.3	5.2 ± 0.5
20:4ω6[f]	tr	2.8 ± 0.2	6.7 ± 0.1	5.6 ± 0.1	37.4 ± 3.4
20:5ω3	3.2	3.2 ± 1.7	1.6 ± 1.1	7.5 ± 4.6	5.3 ± 4.5
22:3ω6	---	---	9.4 ± 1.3	---	tr
22:4ω6	---	2.0 ± 0.6	1.6 ± 0.3	---	13.2 ± 0.7
18:3ω3	---	---	---	32.5 ± 5.1	---
20:4ω3	---	---	---	10.7 ± 0.7	---
22:5ω3	---	1.4 ± 0.4	1.1 ± 0.4	4.5 ± 0.7	1.2 ± 0.0
22:6ω3	---	---	0.8 ± 0.1	---	---
μg Triglyceride per 10^6 cells	1.12	5.94 ± 1.0	7.6 ± 0.8	8.3 ± 0.4	8.6 ± 1.1

[a]At the start of infusion, cells/flask = 1.2 X 10^6; PDL = 32.

[b]Unless noted otherwise, all values are mean ± SD of 3 separate experiments.

[c]A total of 0.4 μmol of each fatty acid was infused into cultures over 72 hr.

[d]Values are mean of 2 experiments.

[e]tr = trace.

[f]20:4ω6 and 20:3ω3 have identical retention times on the packing material used.

TABLE V

Effects of Culture Age[a] and Amount of Fatty Acid Infused[b] on Glycerolipid PUFA Content and Triglyceride Levels of IMR-90 Cells[c]

Culture age	Fatty acid	Amount of fatty acid (μmol)	Fatty acid class composition (wt %)						
			Phospholipid			Triglyceride			Triglyceride (μg per 10^6 cells)
			Saturated	Monoene	Polyene	Saturated	Monoene	Polyene	
32[d]	—	—	28.8	33.0	38.2	67.4	7.9	24.6	1.1
32	18:1	0.4	24.2 ± 0.1	57.0 ± 1.3	18.9 ± 1.3	25.2 ± 3.8	55.0 ±2.8	19.8 ± 6.6	5.9 ± 1.4
32	18:3ω6	0.4	31.5 ± 0.3	19.0 ± 0.6	49.6 ± 0.9	17.5 ± 0.5	5.2 ± 0.7	80.6 ± 2.8	7.6 ± 0.6
32	20:4ω6	0.4	35.8 ± 0.8	23.4 ± 0.2	40.8 ± 0.6	21.8 ± 1.1	8.6 ± 2.8	69.6 ± 3.9	8.6 ± 1.1
53[d]	—	—	29.5	27.4	43.1	59.3	29.6	11.1	3.6
53	18:1	0.4	26.3 ± 3.4	49.6 ± 0.8	24.1 ± 4.2	16.4 ± 0.7	66.8 ± 1.3	17.1 ± 0.3	9.9 ± 0.4
53	18:3ω6	0.4	31.8 ± 5.0	20.2 ± 2.8	48.0 ± 7.8	12.4 ± 1.4	10.2 ± 0.1	77.7 ± 1.6	9.4 ± 1.2
53	20:4ω6	0.4	37.3 ± 4.1	25.0 ± 4.1	37.7 ± 3.0	13.0 ± 4.4	14.9 ± 6.5	72.0 ± 11.0	14.6 ± 0.5
24[e]	—	—	30.5	36.8	32.7	73.2	19.8	9.0	0.3
24[e]	18:3ω6	0.8	31.3	16.4	52.3	7.7	5.0	87.3	10.9
24	20:4ω6	0.8	38.2 ± 1	22.0 ± 0.9	39.8 ± 0.1	11.0 ± 1.9	7.2 ± 0.5	81.7 ± 2.4	11.4 ± 0.3
44[d]	—	—	31.9	38.1	30.0	72.0	17.2	10.8	1.7
44[e]	18:3ω6	0.8	35.0	17.4	47.6	18.1	3.9	78.0	7.9
44	20:4ω6	0.8	37.6 ± 2.0	23.4 ± 0.2	39.0 ± 2.2	13.2 ± 0.5	6.8 ± 0.4	80.8 ± 0.8	13.6 ± 0.5
28[d]	—	—	32.3	38.2	29.5	65.4	19.6	14.9	0.6
28	18:1	0.8	22.3 ± 0.1	61.5 ± 1.0	16.2 ± 1.4	12.2 ± 2.5	80.6 ± 1.8	7.3 ± 0.7	11.8 ± 0.2
52[e]	—	—	31.8	31.0	37.2	67.9	32.3	—	0.8
52	18:1	0.8	21.0 ± 0.1	57.8 ± 0.3	21.2 ± 0.4	11.5 ± 0.6	79.4	9.2 ± 0.6	7.9 ± 1.0

[a]Culture age is given as PDL, the mean number of population doublings since the culture was initiated.

[b]Either 0.4 or 0.8 μmol of fatty acid were infused into cultures. Initial cell number/flask = 0.8-1.2 x 10^6.

[c]Unless noted otherwise, all values are the mean ± SD of 3 separate experiments.

[d]Values are mean of 2 experiments.

[e]Values are from a single experiment.

sufficient exogenous fatty acid to elevate cell triglyceride levels by a factor of 10, while increasing the PUFA composition of that lipid class to twice that of the phospholipid. The fatty acid sequestered in triglyceride during infusion was available for phospholipid synthesis as shown by changing the medium at the end of the infusion period and allowing the cells to continue in culture for an additional 3 days. At the end of the infusion period and 3 days later, the percentage of PUFA in phospholipid was virtually identical despite an increase in cell number by a factor of 2 (Table VI). The amount of triglyceride/cell during this same 3-day interval decreased by a factor of 5 as its PUFA composition decreased from 75 to 35%.

DISCUSSION

The addition of oleic acid to fibroblasts from human skin (GM-10) adversely affected their growth in vitro (1). More recently, using diploid cells derived from primary cultures of human foreskin, the addition of PUFA, especially 20:4ω6, caused suppression of cell growth which was dependent on the concentration of exogenous fatty acid (2). In the same study, 18:1ω9 was without effect, and 18:3ω6 had an effect intermediate between that of 18:1 and 20:4. In this investigation, using amounts of fatty acid similar to those in the study just mentioned (2), the decrease in growth accompanying the addition of PUFA as a single dose at the start of a 72-hr incubation period was greatly reduced if, instead, the PUFA were administered at a constant rate throughout the 72 hr. The difference in triglyceride accumulation at the end of the incubation was negligible; observations by phase microscopy, however, showed that by using the infusion technique, the early accumulation of triglyceride droplets in the cytoplasm could be greatly reduced. The triglyceride, which was accumulated, served as a source of acyl groups, some of which were used for phospholipid synthesis when the cells were returned to the control medium after infusion. These results are in agreement with those obtained with a transformed line of fibroblasts (5).

Providing 20:4ω6 to IMR-90 cells by infusion resulted in inhibition of growth. Unlike the previous study (2), however, doubling the amount of fatty acid supplied did not further depress cell growth. These data suggest that the growth inhibition observed during infusion of PUFA into cultures of low PDL was independent of the nonspecific effects of the exogenous PUFA or of products arising from their autoxidation in the medium. Instead, the exogenous 20:4ω6 or its peroxidation products in the cell may effect a depression of cell growth through their role either as precursors (20) or activators (21), respectively, of prostaglandin biosynthesis. No greater growth inhibition was observed when arachidonic acid levels were doubled, which suggests that the pathways of prostaglandin biosynthesis were saturated or maximally active at the lower level of 20:4. That 18:3ω6 is less effective in suppressing growth may be explained if the rate of converting 18:3ω6 or 20:4ω6 by these cells is slow enough to limit the production of the relevant prostaglandin. In fact, the ratio of 18:3/20:4 increases as the amount of 18:3 supplied is increased, suggesting restricted conversion of 18:3 to 20:4. Regardless of the mechanism, the factors responsible for inhibiting growth of IMR-90 cells become markedly less significant at high PDL. The

TABLE VI

Utilization of Triglyceride Acyl Groups for Phospholipid Synthesis after Transfer of Cells to Fresh Medium Following Infusion with γ Linolenic Acid[a]

Fatty acid[b] infused	Time of harvest[c]		Fraction of acyl groups as polyenoic acids (wt %)		Triglyceride
	End of infusion	3 days post-infusion	Phospholipid	Triglyceride	μg per 10^6 cells
None	+	–	34.0 ± 0.3	–	3.1 ± 0.2
γ18:3	+	–	44.0 ± 0.3	75.9 ± 0.8	35.1 ± 3.5
None	–	+	34.2 ± 0.7	–	2.9 ± 0.3
γ18:3	–	+	44.4 ± 3.0	35.3 ± 4.4	7.2 ± 0.9

[a]All values are mean ± SD of 3 separate experiments.

[b]Cells at an initial density of 1.2 x 10^6/flask were infused for 72 hr with γ18:3 or no fatty acid.

[c]At the end of infusion, half of the cultures were harvested for analysis. Medium was changed in the remaining half, and the cultures incubated an additional 3 days prior to harvest.

reasons for this age-dependent difference are unknown.

Results from these studies suggest the operation of mechanisms which act to prevent PUFA levels from decreasing below 15% or increasing above 50%. Similar limits have been reported for human skin fibroblasts (2) and transformed mouse fibroblasts (18). Limitations on PUFA content may be dictated by the specificities of transacylating enzymes which determine the fatty acid composition in positions 1 and 2 of phospholipid (22). Even with large amounts of either 18:1 or PUFA available within the cell, rapid exchange reactions may prevent a net change greater than that observed in this study. It is unknown whether adaptive changes in the activity of transacylating enzymes are necessary for resistance to extreme changes in the PUFA composition of cell membrane phospholipids. The difference between $\omega 3$ and $\omega 6$ isomers of 18:3 with respect to their elongation and incorporation into phospholipids, as opposed to triglyceride, was not reported in an earlier study (2) in which the $\omega 3$ isomer was employed. Features of shape or packing density may be important in dictating the use of these fatty acids and their elongation products for membrane phospholipid synthesis.

For purposes of comparing the effects of phospholipid PUFA content on the structure and/or function of human diploid cells in culture, the best contrast is produced when cells from cultures infused with 18:3$\omega 6$ are compared with those from cultures infused with 18:1. The PUFA content of membrane lipids in these 2 groups of cells differs by a factor of 3-4. Both fatty acids are incorporated to the same extent into the glycerolipid of cells at high and low PDL and are therefore suitable for long-term studies on the effect of membrane unsaturation on diploid cell function throughout their lifespans in vitro.

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Changes in Host Animal Plasma Lipids during Hepatoma Growth[1]

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ABSTRACT

The concentrations of the major neutral lipid and phospholipid classes in the plasma of rats bearing hepatoma 7288CTC were determined at various times after transplantation. The fatty acid composition of each lipid class was also analyzed quantitatively as tumor growth progressed. Generally, most lipid classes exhibited a slight decrease between the third and sixth day after transplantation, returned to near normal levels by the 15th day, increased dramatically and peaked between the 24th and 27th days before plummeting sharply. At peak concentrations, triglycerides were increased 5 times the normal levels, whereas cholesterol, cholesteryl esters and phosphatidylcholines were increased 3-fold. The percentage of hexadecenoates decreased in all lipid classes as tumor growth progressed and generally, stearate levels increased. In addition to monounsaturated fatty acids, lysophosphatidylcholines and phosphatidylcholines showed relatively large decreases in the percentages of polyunsaturated fatty acids with increased tumor growth. These results indicate that hepatoma 7288CTC can cause perturbation of host animal plasma lipids in the early stages of growth which precedes the massive hyperlipidemia. The interpretation of these results suggests that the early changes in plasma lipids may result from alterations in the normal lipid metabolism of the host, and the hyperlipidemia that develops later may result from the mobilization of lipids to compensate for the altered metabolism.

INTRODUCTION

Hyperlipidemia is generally characteristic of experimental animals bearing tumors, but is less common in human cancer patients. Depletion of carcass lipids in the advanced stages of tumor growth is, however, observed in both humans and experimental animals. An extensive review of the subject prior to 1956 has been compiled by Haven and Bloor (1). Since then, the phenomenon of hyperlipidemia or loss of carcass lipids, or both, has been well documented in laboratory animals (2-5) and humans (6-8). Despite the well recognized effect the tumor has on the lipids of the host, the biochemical events responsible for lipid mobilization have not been identified. Further, few studies have been aimed at determining at what stage of tumor growth hyperlipidemia occurs. This study was aimed at determining the concentrations of individual plasma lipids with progressive growth of Morris minimal deviation hepatoma 7288CTC. A preliminary report of the data has appeared (9).

MATERIALS AND METHODS

A group of 44 male Buffalo strain rats (225-250 g) were implanted bilaterally in the hind limbs with Morris minimal deviation hepatoma 7288CTC and maintained on Wayne Lab Blox laboratory animal chow, whose lipid composition has been reported (10). Control animals were sacrificed directly after transplantation. Groups of 4 animals were sacrificed at 3-day intervals for 30 days. The tumor-bearing rats, hereafter referred to as host animals, were anesthesized with ether and the blood withdrawn from the inferior vena cava with a heparinized syringe. The blood was centrifuged and the plasma stored at -70 C until analyzed. Except where specifically indicated, each sample was analyzed individually.

One ml of plasma was extracted twice using the Bligh and Dyer procedure (11) and separated into neutral lipid and phospholipid fractions with silicic acid (12). The quantities of these lipid fractions were measured gravimetrically. Neutral lipid classes were quantitated by high temperature gas liquid chromatography (GLC) analysis of the intact lipids as described previously (13). Aliquots of plasma phospholipids from individual samples were pooled for each time period for class quantification. Phospholipid classes were resolved by thin layer chromatography (TLC) on adsorbent layers of Silica Gel HR, developed in a solvent system of chloroform/methanol/acetic acid/saline, 50:25:8:4, v/v. Individual classes were quantitated by the Rouser et al. phosphorus analysis procedure (14). Chromatoplates were sprayed with sulfuric acid and charred for visualization and documentation by photography. Individual lipid classes to be used for

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further analyses were visualized with rhodamine 6G or 2',7'-dichlorofluorescein, adsorbent scraped from the chromatoplate and the lipid eluted with solvent.

Methyl esters were prepared from the lipid classes by sulfuric acid-catalyzed transesterification (15) and analyzed quantitatively by analytical GLC using glass columns packed with 10% SP2330 coated on 100-120 mesh chromosorb WAW (Supelco, Inc., Bellefonte, PA). Columns were programmed from 140-220 C at 3 C/min using a Varian Model 3700 chromatograph. Peaks were quantitated using a Spectra-Physics (Santa Clara, CA) digital integrator. Peak identities were based on cochromatography with commercially available standards analyzed on polar and nonpolar columns before and after hydrogenation and analysis of bands resolved according to degree of unsaturation by silver ion TLC.

Glass distilled solvents used in the study were purchased from Burdick and Jackson Laboratories (Muskegon, MI). Lipid standards and chromatography supplies were from Supelco, Inc. (Bellefonte, PA) and Nu-Chek-Prep. Inc. (Elysian, MN).

RESULTS

The concentrations of the major neutral lipid classes found in the plasma of host animals at various times after hepatoma transplantation are given in Table I. Except for cholesterol, the concentration of the lipid classes decreased slightly in the first few days after transplantation and then returned to near normal levels around the 15th day. Beginning around the 15th to 18th day, concentrations increased rapidly and peaked between the 24th and 27th days, before dropping sharply. Cholesterol exhibited a steady increase in concentration that peaked at day 27, 3 times the normal level. Cholesteryl ester concentrations also tripled, but did not show the decrease at day 30 exhibited by the other plasma neutral lipid classes. Triglyceride concentrations peaked at 4-5 times control levels. Because some animals exhibited early hyperlipidemia whereas others were delayed by a day or 2, the variation of concentrations on the 18th, 24th and 30th days was considerable in many instances. This reduced the number of days that the hyperlipidemia differed significantly from control values. The data, however, leave no doubt that hyperlipidemia occurs and involves most neutral lipid classes.

The concentrations of the major phospholipid classes in plasma of host animals at various times after hepatoma transplantation are given in Table II. The changes associated with progressive tumor growth were less dramatic than in the neutral lipids. Most phospholipid classes

TABLE I

Concentration of Plasma Neutral Lipids from Host Animals at Various Times after Hepatoma Transplantation

Days after transplt.	Neutral lipid classes (μg/ml plasma)[a]					
	CHOL[b]	FFA	DG	TG	CE	Total neutral lipid
0	224	176	20	804	470	1781
3	262	54[f]	22	831	383	1566
6	247	69[g]	14	558[e]	411	1292[f]
9	306[d]	190	18	663[d]	570[d]	1981
12	268	118[d]	22	700	469	1628
15	301[c]	286[d]	13[d]	835	484	1973
18	374[f]	408[c]	56[f]	2034[e]	568	3370[e]
21	415[f]	387[e]	62[g]	2174	725[d]	3445[d]
24	571[g]	318[g]	104[g]	3011[g]	948[g]	4953[g]
27	665[g]	227[c]	165[g]	3996[g]	1047[g]	8095[f]
30	625[g]	80[g]	13	766	1229[g]	2713[d]

[a]Concentrations represent the mean of 3-4 animals, except day 30 which consists of 2 animals.

[b]Abbreviations are CHOL = cholesterol; FFA = free fatty acids; DG = diglycerides; TG = triglycerides; CE = cholesteryl esters.

[c]Significance ≥ 0.1 from day zero.

[d]Significance ≥ 0.05 from day zero.

[e]Significance ≥ 0.025 from day zero.

[f]Significance ≥ 0.010 from day zero.

[g]Significance ≥ 0.005 from day zero.

showed increased concentrations in plasma after the 15th day and continuing until they peaked on the 24th to 27th day. Phosphatidylcholine, sphingomyelin and lysophosphatidylcholine, the major plasma phospholipid classes, approximately doubled in concentration over zero-day controls.

The fatty acid compositions of the free fatty acids, triglycerides and cholesteryl esters obtained from host animals at various times after hepatoma transplantation are given in Tables III, IV and V, respectively. Generally, the percentage compositions of the plasma free fatty acids and triglycerides were similar and showed only minimal change in composition with progressive tumor growth. The hexadecenoates of both classes exhibited a gradual decrease over the 30-day period. The high percentage of palmitate and the low level of 18:2 in the plasma triglycerides at the 15th day corresponded to parallel high and low percentages of these 2 acids in the free fatty acid fraction on the same day, suggesting a possible relationship. The high percentages of stearate in the free fatty acids at days 3, 6 and 30 also correspond to the days of low concentrations of this class (Table I). Sterol esters (Table V) contained ca. one-half the percentage of palmitate and 20:4 was a major fatty acid, compared to the free fatty acid and triglyceride fractions (Tables III and IV). Sterol ester hexadecenoates decreased steadily with progressive tumor growth, as was observed for the free fatty acid and triglyceride fractions. Beginning at day 18, 22:6 fatty acid appeared in the sterol esters and the percentage continued to rise for the remainder of the growth period. At day 30, 18:1 and 20:4 percentages dropped to their lowest level and 18:2 incresed to its highest percentage. Day 30 was the time period that the plasma contained the highest concentration of sterol esters (Table I).

The fatty acid compositions of plasma lysophosphatidylcholines and phosphatidylcholines derived from host animals at various times after hepatoma transplantation are given in Tables VI and VII, respectively. Lysophosphatidylcholines showed the largest percentage change during tumor growth of any plasma lipid classes. Mono- and polyunsaturated fatty acids represented more than 30% of the total at the early time periods, but decreased as tumor growth progressed and disappeared completely by day 30. Palmitate percentages remained relatively constant during tumor growth, whereas stearate percentages doubled by day 15 and remained high for the remainder of the experiment. The saturated fatty acids of plasma phosphatidylcholine exhibited a similar behavior to lysophosphatidylcholines: palmitate percentages remained relatively constant, whereas stearate percentage increased moderately until the 15th day and then remained mostly unchanged for the duration of the growth period. The monounsaturated fatty acids showed slight decreases, but the polyunsaturated fatty acids of the phosphatidylcholines showed more dramatic decreases as tumor growth progressed.

DISCUSSION

Analysis of plasma or serum, because of its convenience, is the primary source of data used

TABLE II

Concentration of Plasma Phospholipid Classes in Host Rats at Various Times after Hepatoma Transplantation

Days after transplt.	Phospholipid classes (μg/ml plasma)[a]						
	Lyso-PC[b]	SPH	PC	PI	PS+PE	SF	Total
0	99	75	417	34	15	22	662
3	157	75	512	43	31	22	840
6	104	60	386	17	15	22	604
9	110	75	543	43	23	22	816
12	136	82	512	43	31	22	826
15	104	105	543	34	8	15	809
18	136	157	567	34	23	15	932
21	120	112	732	51	38	7	1060
24	172	165	788	34	54	0	1213
27	151	142	1322	77	61	22	1775
30	115	120	858	43	61	0	1197

[a]Values represent the mean of duplicate determinations of a pooled sample from 4 animals, except days 24 and 27 where 3 animals were pooled and day 30 which consisted of 2 animals.

[b]Abbreviations are Lyso-PC = lysophosphatidylcholine; SPH = sphingomyelin; PC = phosphatidylcholine; PI = phosphatidylinositol; PS = phosphatidylserine; PE = phosphatidylethanolamine; SF = solvent front.

TABLE III

Fatty Acid Composition of Plasma Free Fatty Acids Derived from Animals at Various Times after Hepatoma Transplantation

Days after transplt.	Fatty acid percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
0	28.8	6.4	5.6	25.8	22.2	1.6	0.8
3	42.5	2.9	20.3	24.9	8.6	-	-
6	40.3	2.2	17.6	26.0	12.3	-	-
9	34.8	6.4	7.3	26.6	19.5	1.3	-
12	39.6	5.5	8.8	26.6	15.8	0.7	-
15	40.9	4.9	8.8	28.3	9.6	0.8	0.6
18	30.4	3.8	5.6	29.7	25.4	1.1	0.5
21	31.7	3.4	6.1	32.4	22.2	0.7	0.3
24	30.2	3.2	7.8	32.4	21.7	0.9	0.4
27	31.9	3.1	6.8	26.1	25.9	1.1	0.8
30	31.9	1.8	17.6	19.9	18.2	-	-

[a]Percentages represent the mean of 3 or 4 animals. The differences between the sum of the percentages and 100% in any row represents the sum of other fatty acids present in small amounts but not shown in the table.

TABLE IV

Fatty Acid Composition of Plasma Triglycerides Derived from Animals at Various Times after Hepatoma Transplantation

Days after transplt.	Fatty acid percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
0	25.2	7.0	2.2	29.8	25.2	1.6	1.8
3	29.2	6.4	3.7	27.8	22.8	1.6	0.8
6	31.0	4.0	3.3	28.2	27.1	1.2	0.6
9	27.7	3.6	3.4	28.2	30.0	1.6	1.0
12	30.0	3.6	4.5	29.1	23.8	1.1	0.6
15	39.6	2.8	4.8	31.9	18.3	1.1	-
18	29.0	2.6	3.8	27.3	28.3	2.8	1.1
21	27.2	2.3	3.6	28.3	28.6	3.2	1.8
24	29.9	1.7	4.4	29.4	27.2	2.5	1.6
27	28.9	2.2	3.6	26.2	30.2	2.0	1.6
30	30.4	1.6	4.9	26.2	31.3	1.9	1.1

[a]Percentages represent the mean of 3 or 4 analyses. The differences between the sum of the percentages in any row and 100% represent the sum of other fatty acids present in small amounts but not shown in the table.

TABLE V

Fatty Acid Composition of Plasma Sterol Esters Derived from Animals at Various Times after Hepatoma Transplantation

Days after transplt.	Fatty acid percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
0	15.5	7.0	0.3	13.6	32.3	29.6	-
3	16.5	6.6	0.9	10.3	35.2	27.8	-
6	15.5	1.9	0.8	8.9	37.8	33.5	-
9	14.3	2.0	1.0	11.4	32.1	38.8	-
12	16.0	2.6	1.2	13.2	28.4	38.0	-
15	19.8	2.9	1.8	17.2	25.0	33.0	-
18	17.8	2.1	1.9	15.9	26.1	35.3	0.6
21	16.1	1.8	2.1	13.2	27.6	37.2	1.0
24	14.4	1.1	1.9	14.8	28.6	35.1	3.4
27	15.4	1.4	2.1	10.2	31.9	35.1	2.6
30	15.2	0.9	0.8	8.6	43.3	26.5	3.2

[a]Percentages represent the mean of 3 or 4 analyses. The differences between the sum of the percentages in any row and 100% represent the sum of other fatty acids present in small amounts but not shown in the table.

to monitor the nutritional and health status of an animal. The interpretation of the data from plasma is complicated because the data represent the sum total of numerous metabolic processes. The analysis of plasma lipids from a tumor-bearing animal is no exception and even adds to the complexity. Plasma lipid class concentrations and compositions represent the sum total of lipids contributed by: (a) endogenous biosynthesis; (b) catabolism; (c) diet; (d) mobilization of stored fat; and (e) the tumor itself. At the early stages of tumor growth, diet, fat mobilization and the tumor probably have a negligible effect; but in the later stages of tumor growth, when diet consumption is reduced, adipose tissue is being depleted and tumor mass has increased significantly, these sources of plasma lipids probably play a major role. Despite multiple sources of plasma lipids that change as tumor growth progresses, lipid class concentration and composition profiles for the complete growth period may contain some useful information, especially at the early stages of tumor growth.

Few studies have followed the changes in individual lipid classes of the plasma from the host animal during tumor development. Brenneman et al. (2) have shown that host animals bearing Ehrlich ascites carcinoma cells developed a pronounced hypertriglyceridemia and a slight hypercholesterolemia that peaked at mid-growth period and then declined to near normal levels at termination. Hypertriglyceridemia occurred much sooner and fell more slowly than we observed for host animals of hepatoma 7288CTC. Cholesterol ester and triglyceride concentrations in whole blood from host animals bearing hepatoma 7777 have been reported at 4 time-periods between day 22 and 58 after transplantation (16). A 3-fold increase in cholesterol esters occurred before the 50th day, but the increase was more gradual than we observed in our study. They concluded that the rise in blood cholesterol and cholesteryl esters was caused by the release of cholesterol by the tumor. Because of the magnitude of the abrupt rise in cholesteryl esters after the 18th day (Table I), it would appear doubtful that the hepatoma 7288CTC was the major source of cholesterol in our study. In contrast to the hypertriglyceridemia we observed and for host animals of Ehrlich ascites cells (2), they reported a 50% decrease in blood triglyceride levels of hepatoma 7777-bearing animals between the 22nd and 35th days. These comparisons make it clear that the hyperlipidemia which develops in tumor-bearing animals may depend on the elevation of one or more lipid classes and that the onset, duration, amplitude and decline of the hyperlipidemia depends on the type of tumor. The data also indicate the necessity of determining individual plasma lipid class profiles over the entire tumor growth period. Had we looked at host plasma lipid levels before day 18 and after day 27, hyperlipidemia would not have been observed.

A close look at the concentration and the fatty acid composition profiles of the major lipid classes in host plasma as hepatoma growth progressed is of interest. At the earliest time examined after tumor transplantation, free fatty acid concentrations were one-third zero-day values and by day 6 significant hypolipidemia of total neutral lipids was observed (Table I). The hypolipidemia can very easily be overlooked because of the magnitude of the hyperlipidemia that develops later. The early hypolipidemia may result from first alteration, interruption, or inhibition of certain lipid metabolic steps in the host animal brought about by the hepatoma. One such metabolic step appears to be the inhibition of desaturation. We have shown in a cell-free system that the $\Delta 9$ desaturase system decreases abruptly in host liver after hepatoma transplantation (17). Mapes and Wood (18) have shown that oleate represented a higher percentage of the octadecenoates isolated from host animal plasma phospholipids as early as 3 days after transplantation and that the changes were highly significant at 6 days. The increased proportion of oleate could have resulted from a decrease in the synthesis of vaccenate. Since it is known that palmitoleate is the precursor of vaccenate in liver (19) and the hepatoma (20), this view is supported by the observed decrease in the percentage of the hexadecenoates in all the lipid classes beginning at the earliest time and continuing for the duration of tumor growth (Table III-VII). The decreased hexadecenoate level probably resulted from the inhibition of the $\Delta 9$ desaturase system. The increased percentages of stearate and the decreased levels of the octadecenoates in the plasma phospholipids (Tables VI and VII) are consistent with this line of reasoning. The plasma neutral lipid classes showed slight increases in stearate, but little or no change in the octadecenoate percentages. The lack of a decrease in the octadecenoate percentages can be explained by the mobilization of adipose rich in oleate and by the apparent existence of an alternate route of oleate biosynthesis (21-23). The reason for the decreased desaturase activity is not apparent at this time. One is tempted to speculate that a reduced level of NADPH might be responsible. However, a comparison of tissue level of NADPH (24) and desaturase activities (17,25)

indicates a lack of correlation.

It is possible that the inhibition of desaturation includes the polyunsaturated fatty acids. Nearly 40 years ago, Smedley-Maclean and colleagues (26,27) reported that the Walker Tumor reduced the level of polyunsaturated fatty acids in the surrounding tissue. The observed decrease of the polyunsaturated fatty acids in plasma phospholipids (Tables VI and VII) with increased tumor growth is consistent with the possible inhibition of polyunsaturated fatty acid biosynthesis, but is also consistent with the possible removal of these acids by the tumor. The unchanged to marginally higher percentages of polyunsaturated fatty acids in the neutral lipids (Tables III-V) might appear contradictory, but it is possible that these classes may represent active forms of mobilization.

The change from hypolipidemia to hyperlipidemia after 12-15 days of tumor growth may represent the host's response to compensate for initial effects on lipid metabolism exerted by the hepatoma. This response may involve the mobilization of reserve fat stores to supply one or more of the fatty acids or complex lipids required by the host, but whose concentrations have fallen as a result of the tumor. It is also possible that the tumor produces a fat mobilization substance directly. If this were the case, one might expect to see a gradual increase in all the lipid class concentrations much like that observed for free cholesterol (Table I) and hypolipidemia would not be expected. Whether the fat mobilization substance(s) are produced directly by the tumor or whether the host is stimulated to produce such substances by the tumor is not

TABLE VI

Fatty Acid Composition of Plasma Lysophosphatidylcholine Derived from Animals at Various Times after Hepatoma Transplantation

Days after transplt.	Fatty acid percentages[a]					
	16:0	16:1	18:0	18:1	18:2	20:4
0	38.1	1.4	24.1	16.1	16.6	2.5
3	36.6	1.0	28.6	12.1	18.0	2.3
6	37.8	1.0	30.0	11.0	12.5	2.2
9	37.2	1.4	42.5	8.2	6.3	1.6
12	37.4	0.9	43.8	7.5	5.9	1.9
15	35.9	1.4	51.1	7.4	1.2	0.2
18	36.3	1.0	53.8	2.6	0.3	0.3
21	38.0	-	54.3	4.6	1.3	-
24	33.0	-	59.4	4.1	0.3	-
27	38.0	-	54.1	3.2	-	-
30	32.8	-	56.6	-	-	-

[a]Percentages represent the mean of 2 or more analyses. The differences between the sum of the percentages in any row and 100% represents the sum of other fatty acids present in small amounts but not shown in the table.

TABLE VII

Fatty Acid Composition of Plasma Phosphatidylcholine Derived from Animals at Various Times after Hepatoma Transplantation

Days after transplt.	Fatty acid percentages[a]						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
0	27.1	1.6	22.4	13.8	21.6	8.4	-
3	26.6	1.0	22.8	10.6	26.3	8.2	0.9
6	30.0	-	24.2	9.3	30.0	5.7	-
9	24.7	0.6	26.1	9.6	26.2	10.2	0.8
12	27.0	0.6	27.3	8.6	23.3	10.4	1.0
15	35.6	0.5	35.0	10.2	12.6	4.1	0.4
18	35.6	0.6	33.9	9.3	14.7	3.2	-
21	31.7	0.4	33.6	10.0	17.0	4.8	0.8
24	31.1	-	28.0	16.6	19.9	2.7	0.6
27	31.1	0.3	35.8	8.0	14.6	4.4	-
30	39.8	0.4	32.8	8.2	13.3	1.1	-

[a]Percentages represent the mean of 3 or 4 analyses. The differences between the sum of the percentages in any row and 100% represent the sum of other acids present in small amounts but not shown in the table.

known at this time, but there appears to be ample evidence that one or more such compounds are produced. Bizzi et al. (28) have suggested that the Walker 256 carcinosarcoma produces a substance responsible for the mobilization of adipose tissue. More recent work with this tumor has shown that the rates of lipolysis in the adipose tissue of host animals were 2-3 times higher than control animals, but these workers (4) were unable to detect any stimulatory factor in serum. Recently Kitada et al. (5) have shown, using a new approach, that serum from lymphoma-bearing AKR mice contains a factor that produces massive fat mobilization in normal animals. These preliminary data appear to demonstrate that the lymphoma, and perhaps other tumors as well, produce a substance or substances that are directly or indirectly involved in the mobilization of fat reserves in the host animal. We are suggesting on the basis of the data presented in this paper and other reports that the fat mobilization may be a response by the host to compensate for the interruption of some normal lipid metabolic processes produced by the tumor. This is a working hypothesis that other investigators might find useful.

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Lecithin Influence on Hyperlipemia in Rhesus Monkeys

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ABSTRACT

Previous studies in humans have shown that the ingestion of lecithin can alter plasma cholesterol and triglyceride concentrations by mechanism(s) that remain to be elucidated. To further explore this response to lecithin, hyperlipemic rhesus monkeys were selected from a group of animals fed a semi-purified diet containing corn oil, casein, sucrose and cholesterol (120 mg/100 Kcal) for 10 years. Soybean lecithin (92% phosphatidylcholine) was supplemented in the diet (0.5 g/100 Kcal) of these monkeys. Measurements of plasma cholesterol, triglycerides and phospholipid were made prior to, during and following 7 wk of lecithin supplementation. In addition, determinations of triglyceride secretion rates following administration of Triton WR1339, triglyceride clearance after intravenous infusion of Intralipid® and plasma lecithin:cholesterol acyl transferase enzyme (LCAT) activity were assessed at the same time intervals. As in other studies, manipulation of lecithin intake elicited a highly variable response, but significant changes were observed in plasma cholesterol and triglycerides as a consequence of supplementing or removing lecithin from the diet. Lecithin had no influence on the absolute plasma phospholipid level or LCAT activity. However, lecithin significantly reduced total lipids, increased the relative concentration of phospholipid and tended to increase the phospholipid/free cholesterol (PL/FC) concentration. While lecithin did not significantly affect triglyceride secretion rates, all animals were able to clear Intralipid® (triglyceride) more efficiently while fed lecithin. These data are interpreted to mean that the reduction in plasma lipids associated with lecithin ingestion may have been mediated via enhanced clearance of lipids transported in lipoproteins of lower density, whereas the rebound folllowing lecithin removal reflected reduced clearance of these lipids.

INTRODUCTION

Hypercholesterolemia and hypertriglyceridemia are risk factors for atherosclerosis (1) that respond variably to dietary modification of fat and cholesterol (2,3). Although polyunsaturated fat is generally hypocholesterolemic in man, the response is variable among individuals. Similarly, polyunsaturated dietary phosphatidylcholine (PC), or soybean lecithin, often elicits a hypocholesterolemic effect, a response that has been reported to be both separate from its polyunsaturated or essential fatty acids (4) yet dependent upon the fact that it is an unsaturated lecithin (5). Investigation of the lecithin effect on lipemia and experimental atherosclerosis have also generated conflicting results (6-8). These differences may be attributable to variations in the unsaturation or amount of lecithin used and its mode of administration, the clinical characteristics of test patients or species of animal and the parameters assessed. In an attempt to resolve this confusion and explore the mechanism(s) of lecithin action, a group of hyperlipemic rhesus monkeys fed a sucrose, polyunsaturated fatty acid (PUFA) and cholesterol-containing diet for several years was studied.

Enhanced clearance of circulating lipid as a possible mode of action for lecithin was supported by the reduction in low density lipopoprotein (LDL) concentration following lecithin supplementation of humans (4,9). Clark (10) has shown that introduodenal infusion of lecithin in rats reduced cholesterol ester transport from the gut, possibly via phospholipid changes in the chylomicron surface coat. In addition, Tall and Small (11) have postulated a mechanism linking chylomicron clearance with high density lipoprotein (HDL) particle formation that depends on adequate phospholipid and surface apoproteins associated with the chylomicron.

Thus, on the assumption that the diet-induced hyperlipemia in these rhesus monkeys may have been associated with impaired lipoprotein clearance similar to that observed in rabbits (12) and monkeys (13,14) and from the data on humans (7) which implicated the lecithin:cholesterol acyl transferase enzyme (LCAT) (EC 2.31.43) in lipoprotein cholesterol clearance, we examined the possibility that lecithin may have been a limiting factor for efficient lipoprotein metabolism in these monkeys.

METHODS

Seven female rhesus monkeys *(Macaca mulatta)* ca. 12 years of age which had been fed a semipurified diet containing all carbohydrate as sucrose, 8% corn oil and ca. 0.5% cholesterol (120 mg/100 kcal) (Table I) for 10 years were chosen for this study. The monkeys were

characterized as hyperlipemic (average plasma cholesterol and triglyceride values were 296 and 188 mg/dl, respectively) (Table II) and were considered useful models for study of lecithin supplementation since the corn oil diet minimized the possibility that any hypolipemic effect of lecithin would be attributed to its PUFA content.

To test various plasma lipid parameters as a function of lecithin supplementation, analyses were performed before, 7 wk after initiation of lecithin and 16 wk following lecithin removal from the diet, which was fed ad libitum throughout the study. The level of purified soybean lecithin added (Phospholipon 100®, Natterman) (Table I) represented 0.5 g/100 Kcal of diet or the equivalent of 10-12 g/day in humans consuming 2000-2400 calories. Blood collections and injections were made through a catheterized popliteal vein of fully conscious monkeys trained to sit in primate restraining chairs. A single fasting blood sample from each animal was collected in EDTA (1 mg/ml) and plasma was separated by centrifugation at 4 C for measurement of total cholesterol (15) as well as the distribution of cholesterol between plasma LDL and HDL by heparin-manganese precipitation (16). The modifications adopted in the precipitation procedure include a final concentration of manganese of .136 M, heparin at 3.2 mg/ml and 10-min incubations of plasma at room temperature. Plasma triglycerides (Kit #320A, Sigma Chemical Co., St. Louis, MO) and total phospholipids (17) were also assayed. Free cholesterol and LCAT activity were measured (18), the LCAT by a modified Stokke-Norum method which measures the conversion of radiolabeled cholesterol to cholesteryl ester. This assay estimated both the percentage and molar rates of cholesterol esterification in total plasma. Measurements of fat clearance (19) were determined in fasted, chaired monkeys by intravenous injection of Intralipid® (Vitrium, Stockholm, Sweden) at a dose of 675 mg/kg body weight followed by measures of plasma triglyceride at 2, 5, 10, 20, 30, 40, 60, 90, 120 and 150 min intervals. Twenty-four hr later, hepatic triglyceride secretion rates (TGSR) were obtained following administration of Triton WR1339 at a dose of 250 mg/kg body weight (20). Data were analyzed by the paired, 2-tailed "t" test.

TABLE I

Experimental Diet Fed to Rhesus Monkeys

Ingredients	Dry ingredients[a] g/100 g
Casein	21.20
Sucrose	49.10
Corn oil	6.80
Vitamin mix[b]	0.40
Mineral mix[c]	3.60
Choline chloride	0.40
Inositol	0.10
Cholesterol, USP	0.40
Cellulose[d]	18.00
Lecithin[e]	(1.70 g added to 100 g diet during supplementation period)

[a]Agar solution (1.25%) was mixed with solid ingredients at rate of 80 ml/100-g diet.

[b]Vitamin mix contained in g/kg of mix: thiamine hydrochloride, 0.8; riboflavin, 1.6; pyridoxine hydrochloride, 0.8; calcium pantothenate, 5.0; niacinamide, 8.0; folic acid, 0.8; biotin, 0.04; cyanocobalamine, 0.03; menadione, 1.00; DL α-tocopheryl acid acetate (500 IU/gm), 20.00; vitamin A acetate (500,000 IU/gm), 5.00; D_3 (Dura D Beadlets) (200,000 IU/gm), 1.25; ascorbic acid, 122; dextrin, 833.

[c]Mineral mix contained in g/kg of mix: calcium carbonate, 290.12; potassium phosphate dibasic, 311.88; calcium phosphate dibasic, 72.53; magnesium sulfate, 98.64; sodium chloride, 161.99; ferric citrate, 26.59; potassium iodide, 0.77; manganese sulfate, 4.84; zinc chloride, 0.24; cupric sulfate, 0.29; chromium acetate, 0.04; sodium selenite, .0043; magnesium oxide, 34.04.

[d]Alphacel, Bio-Serv., Inc., Frenchtown, NY.

[e]Lecithin (Phospholipon® 100) was kindly supplied by the American Lecithin Co., and was obtained from Nattermann Chemie GmbH, Koln, West Germany. This processed soybean lecithin contained 92% phosphatidylcholine and 4% lysophosphatidylcholine. Analysis indicated that PUFA content exceeded 75% of the total fatty acids.

RESULTS

Body Weight

Minor fluctuations in body weight were observed throughout the study, but mean values did not differ at any time, nor could individual weight loss or gain be correlated with any of the metabolic responses recorded (Table II).

Plasma Cholesterol Values

The initial plasma cholesterol concentration reflected the long-term supplementation of cholesterol and tended to be elevated with a mean of 295 mg/dl with the following distribution: 3 monkeys, 206-225 mg/dl; 3 monkeys, 261-283 mg/dl and 1 monkey, 608 mg/dl. The data in Table II represent the response to lecithin supplementation and removal for all monkeys combined since the percentage change was usually similar except where noted. For instance, lecithin supplementation produced an average reduction in plasma cholesterol concentration of 23% with the greatest absolute decrease (237 mg/dl) occurring in the most hyperlipemic monkey. One monkey

actually increased its plasma cholesterol 10%. After removal of lecithin, the average cholesterol increased 38% above the mean value observed during supplementation, although 2 monkeys had minimal increases of less than 5%.

Lipoprotein Cholesterol Distribution

Lecithin supplementation also affected cholesterol distribution within lipoproteins. HDL cholesterol concentration, which increased 10% with lecithin supplementation, was significantly decreased (28%) following lecithin removal. Lecithin lowered LDL cholesterol levels an average of 36%, the greatest absolute decline (243 mg/dl) again occurred in the most hyperlipemic monkey. Upon removal of lecithin, LDL cholesterol rose significantly by an average of 86%, in which the greatest absolute increase was 194 mg/dl.

The LDL/HDL ratio reflected the predominance of LDL prior to lecithin supplementation, but was subsequently reduced by lecithin feeding because of the large decline in LDL cholesterol. An initial average ratio of 2.39 declined 42%; the greatest drop was again observed in the most hyperlipemic monkey. Removing dietary lecithin resulted in an exaggerated rebound in the ratio of 158% resulting from the expected rise in LDL cholesterol and an unanticipated, exaggerated reduction in HDL cholesterol (Table II).

Plasma Total Lipids, Phospholipids and Free Cholesterol

Since individual lipid classes tended to be elevated, it was not surprising that total lipids values were high. There was no appreciable change in absolute concentrations of plasma phospholipids during the period of lecithin supplementation, whereas total lipids and free cholesterol were reduced. In relationship to the decrease in total lipids, the percentage phospholipid increased significantly; the phospholipid-to-free cholesterol ratio tended to improve (Table II).

Plasma Triglycerides

Initially, elevated triglyceride levels declined an average of 34% in all monkeys fed lecithin with wide variation in the response. Following removal of lecithin, values rose 64% (Table II).

Triglyceride Clearance and TGSR

Lecithin affected Intralipid® clearance by enhancing the removal rate of the infused lipid ca. 30% in most monkeys (Table III). However, fasting, and presumably hepatic, triglyceride secretion rates were unaffected by lecithin supplementation. Measurements of

TABLE II

Concentrations and Relative Shifts in Plasma Lipids before, during and after Supplementation of Hyperlipemic Rhesus Monkeys with Dietary Lecithin

Parameter	Lecithin status					
	Before	During	% Change	After	% Change
Body weight, kg	8.8 ± 0.4	8.8 ± 0.6	-1	8.6 ± 0.6	-3
Total cholesterol, mg/dl	296 ± 53	228 ± 32[a]	-23	316 ± 35[b]	+38
LDL cholesterol, mg/dl	208 ± 56	132 ± 31[a]	-36	246 ± 42[b]	+86
HDL cholesterol, mg/dl	87 ± 9	96 ± 12	+10	69 ± 10[b]	-28
LDL/HDL	2.39 ± 0.88	1.38 ± 0.43[a]	-42	3.56 ± 1.15[b]	+158
Triglyceride, mg/dl	188 ± 38	124 ± 34[b]	-34	204 ± 25[b]	+64
Phospholipids, mg/dl	478 ± 49	440 ± 34	-8	ND	
(% total lipids)	(48.7 ± 1.7)	(57.7 ± 2.5)[b]		ND	
Free cholesterol, mg/dl	103 ± 27	81 ± 17[a]	-21	ND	
Total lipids, mg/dl	977 ± 88	780 ± 91	-20	ND	
PL/FC	5.5 ± 0.7	6.3 ± 0.8	+15	ND	

Values represent mean ± SE for 7 monkeys.

[a]Change differs from previous mean, $P < 0.10$.

[b]Change differs from previous mean, $P < 0.05$.

TABLE III

Intralipid® Clearance, Triglyceride Secretion Rate (TGSR) and Plasma LCAT Activity before and during Supplementation of Hyperlipemic Rhesus Monkeys with Dietary Lecithin

Determination	Lecithin status		
	Before	During	% Change
Intralipid® clearance (t½ min)	127 ± 18	81 ± 13[a]	-36
TGSR (mg/kg/hr)	68 ± 7	73 ± 6	+9
LCAT activity, Percent esterification (% hr)	8.5 ± 1.7	8.9 ± 0.9	+5
Molar rate of esterification (μmol/ℓ/hr)	190 ± 33	176 ± 27	-8

Values represent mean ± SE for 7 monkeys.

[a]Significant change ($P < 0.05$) from previous mean.

triglyceride clearance and secretion were not performed following removal of lecithin from the diet.

LCAT Activity

LCAT activity of the entire group of monkeys (Table III), measured in terms of the fractional rate of esterification and mass of cholesterol esterified, was unaffected by lecithin. However, the most lipemic monkey had an extraordinarily low fractional rate of esterification (2.0%) that increased to normal (8.8%) with lecithin treatment. In addition, the improved fractional rate of esterification for this monkey led to a marked increase in its molar rate of esterification, despite the substantial decline in plasma free cholesterol substrate (Table II). The relatively high initial mean free cholesterol concentration (103 mg/dl) for all monkeys reflected the elevated total plasma cholesterol values, but represented the normal proportion (33%) of the total plasma cholesterol pool. Furthermore, this percentage of free cholesterol remained constant during lecithin supplementation. Accordingly, lecithin induced a significant drop in the absolute mass of circulating free cholesterol.

DISCUSSION

The purpose of this study was to determine whether a specific diet-induced hyperlipemia in rhesus monkeys might respond to dietary lecithin. One advantage of the study design was the opportunity to use each monkey as its own control twice. The positive, but variable, results raise several points concerning possible mechanisms of lecithin involvement.

Lecithin vs PUFA

In this study, the hypolipemic effect of lecithin should not have reflected a limitation in dietary polyunsaturated fat since the dietary fat was corn oil. The fact that lecithin decreased LDL and increased HDL cholesterol concentrations while decreasing triglycerides (presumably VLDL and chylomicron remnants) in monkeys with diverse plasma cholesterol levels implies that, as in human studies (4), the response was specific to lecithin and not necessarily dependent on the hypolipemic action of PUFA derived from the soybean lecithin as suggested elsewhere (21). The PUFA-induced depression in cholesterolemia is thought to correct altered lipoprotein fluidity and clearance because of the saturated fatty acids in cholesteryl esters and phospholipids (22,23) which should not have pertained with corn oil as the dietary fat. Furthermore, the response to polyunsaturated fat is typically associated with a decrease in HDL cholesterol (22,24).

Lecithin and Lipoprotein Interconversions

Lecithin resulted in a reduction of lipoprotein cholesterol similar to that observed in some humans (4), i.e., LDL cholesterol decreased whereas the concentration in HDL cholesterol increased slightly. Since the absolute plasma phospholipid concentration was unchanged whereas total lipid and free cholesterol tended to decline during lecithin supplementation, the relative status of phospholipid to other lipid fractions was enhanced. Following lecithin removal, a reverse shift in lipoprotein cholesterol was noted between apo B-containing lipoproteins and HDL. These shifts argue well for a lecithin-enhanced catabolism and/or transfer of specific components of apo B lipoproteins (VLDL, chylomicrons) to apo A lipoproteins (HDL), a conversion which is presumably dependent upon proper phospholipid/free cholesterol ratios of the triglyceride-rich lipoprotein surface coat and lipoprotein lipase-LCAT interactions (25,26).

For example, Fielding (27) demonstrated that increased lipoprotein free cholesterol, presumably as a surface component, reduced the lipoprotein lipase-associated removal of lipoprotein triglyceride. Surface loading of free cholesterol and depressed lipoprotein lipase activity would follow from delayed remnant clearance that presumably occurs with cholesterol feeding in monkeys (13,14) and rabbits (12). Tall and Small (11) have recently expanded this hypothesis to include a mechanism for the transfer of HDL precursors from surface components of triglyceride-rich lipoprotein during catabolism of the lipoproteins. The inverse relationship between plasma triglycerides and HDL cholesterol, especially following lecithin removal from the diet in this study, also supports this precursor-transfer concept. It is yet to be determined whether lecithin actually effected such a transfer and, if so, how the sucrose-cholesterol feeding induced a limitation in lecithin availability initially.

It is conceivable that dietary cholesterol might alter lipoprotein surface coat. Clark (10) has demonstrated that duodenally infused lecithin in the rat increased the phospholipid/free cholesterol ratio of chylomicrons and depressed the incorporation of cholesterol ester into this gut lipoprotein. On the other hand, cholesterol feeding in rhesus monkeys reportedly increased the free cholesterol concentration of lymph lipoproteins (28). Although we did not isolate and characterize specific lipoproteins in this study, the high level of dietary cholesterol without adequate synthesis or dietary availability of lecithin may have resulted in a decreased phospholipid/free cholesterol ratio of chylomicrons since that ratio tended to be depressed for total plasma lipids. Such a depression could adversely affect lipoprotein lipase clearance of triglyceride (26,27). Another possibility is that dietary lecithin may have countered a deficit in hepatic (or intestinal) phospholipid synthesis brought about by sucrose feeding, a phenomenon which has been reported both in humans (29) and rats (30). Current studies are examining these possibilities.

Lecithin and Triglyceride Removal

The marked reduction in plasma triglyceride in response to lecithin suggested that catabolism of VLDL and/or chylomicrons was enhanced by lecithin or that secretion of triglyceride was reduced. The possibility of lecithin enhancement was strengthened by the increased Intralipid® clearance of lipoprotein lipid. The fact that the triglyceride secretion rate was slightly increased by lecithin implies that altered clearance, and not synthesis or secretion, of triglyceride (presumably VLDL) was a major factor in this diet-induced hyperlipemia.

The pronounced reduction of circulating triglyceride by lecithin was similar to the response in chimpanzees fed lecithin (5). Lecithin supplementation in humans generally is without effect or even increases the plasma triglyceride concentration (6,7,21), except in the case of carbohydrate-induced triglyceridemia where lecithin feeding reduced triglycerides (31). Our monkeys consumed a relatively low-fat (20% of calories), high carbohydrate (53% of calories from sucrose) diet, and the hypertriglyceridemia may have reflected this dietary circumstance since sucrose is known to stimulate fatty acid and triglyceride synthesis while suppressing synthesis of phospholipid (30). The beneficial effects of lecithin during carbohydrate feeding in man was ascribed to the essential fatty acids of lecithin (21,31), which should not have been a factor in this study since, as previously stated, the dietary fat was corn oil. On the other hand, studies of lecithin absorption have revealed that the greatest incorporation of lecithin into chylomicrons occurred when large amounts of corn oil were fed simultaneously (32). Lecithin feeding apparently does not alter the rate of cholesterol absorption in man (22).

Lecithin and LCAT

The failure of lecithin to influence LCAT activity was surprising, since one might expect LCAT activity would vary if clearance of VLDL was altered and/or the phospholipid profile of lipoproteins was enhanced (5,26). Activity of LCAT has been correlated with the serum triglyceride level (33) and the unsaturation of the dietary fat in cebus and squirrel monkeys, but not rhesus monkeys (34,35). With the exception of the hyper-responder monkey, all LCAT values were within the normal range for rhesus monkeys previously studied under similar dietary circumstances (35). It was notable that the severely depressed LCAT activity (fractional rate) of the most hyperlipemic monkey did improve with lecithin supplementation.

Variable Response to Lecithin

An additional point was the variation in response to lecithin, an observation that is reminiscent of human studies (4,7,22,29). It will be important to ascertain what specific factors (e.g., apoproteins, endogenous lecithin synthesis, gut incorporation of cholesterol) influence the lecithin response. The role of diet

must be carefully considered in these studies since dietary lecithin may be effective only when certain dietary constituents, such as cholesterol or sucrose, compromise the normal packaging and metabolism of the lipoproteins.

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Effect of Colestipol on Sterol Metabolism in the Rat

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ABSTRACT

Sterol metabolism studies using isotopic and chromatographic techniques were performed on rats fed diets supplemented with colestipol (Upjohn). Compared to controls, colestipol altered sterol metabolism dramatically. Bile acid output increased from 7.0 mg/day to 12.2 mg/day (0.42% colestipol) and 39.6 mg/day (1.67% colestipol). Daily fecal neutral sterol output and daily endogenous neutral sterol output increased 36% and 55%, respectively, on the 1.67% colestipol diet. Cholesterol absorption was reduced by colestipol feeding. Cholesterol balance increased dramatically with 1.67% colestipol administration (43.5 mg/day vs -1.0 mg/day in controls). Colestipol exerts its effect by binding bile acids and by bile acid depletion interfering with cholesterol absorption.

INTRODUCTION

Colestipol hydrochloride (Colestid, Upjohn Co., Kalamazoo, MI), a bile acid sequestering resin, is a high-molecular weight, insoluble copolymer of tetraethylenepentamine and epichlorohydrin. It has been reported that long-term administration of colestipol effectively lowers serum cholesterol in experimental animals and in man (1-5). This resin binds bile acids in the intestine, prevents their reabsorption and causes a greatly increased fecal excretion of acidic steroids which secondarily results in an increased catabolism and synthesis of cholesterol and a reduced plasma cholesterol level (1,4,5).

In this communication, we present detailed data which shows the effect of colestipol administration on the sterol metabolism in the rat. Sterol measurements using isotopic and chromatographic techniques were carried out by administering low-dose (0.42%) and high-dose (1.67%) colestipol to rats.

MATERIALS AND METHODS

Animals and Diet

Male Sprague-Dawley derived rats weighing 200-225 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 basal diet consisted of Rockland rat chow (19% protein, 10% fat, 3% fiber, 61% carbohydrate and required vitamins and minerals) supplemented with 5% corn oil and contained an average of 0.62 mg/g β-sitosterol. This diet was then further supplemented with 0.1% cholesterol by dissolving this cholesterol in ethanol and mixing it with the food. The alcohol was allowed to evaporate. After complete dryness, this diet was supplemented with either 0.42% colestipol hydrochloride (for the low-dose colestipol group) or 1.67% colestipol hydrochloride (for the high-dose colestipol group) and was thoroughly mixed.

On the first day of the experimental period, each animal was given an intraperitoneal injection of 10 μCi of DL-[2-^{14}C] mevalonolactone; feeding of the experimental diet was begun several hours later. Control rats were always studied concurrently with the colestipol-fed groups and were maintained under identical conditions. Feces were collected in 3 2-day pools on days 10, 12 and 14 after isotopic labeling and were dried, weighed and ground in a mortar for subsequent neutral sterol and bile acid analyses. Beginning on day 4 of the experimental period and every 2 days thereafter, blood was obtained from the tail vein for determination of plasma cholesterol concentration and plasma cholesterol specific activity (sp act). At the end of the 14-day experiment, 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. A section of the liver was excised for determination of liver cholesterol concentration and sp act as described previously (6,7).

Labeled Compounds

DL-[2-^{14}C]-Mevalonolactone in benzene (Amersham-Searle, Arlington Heights, IL) was found to be greater than 95% pure on silica gel thin layer chromatography (TLC) with acetone/

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benzene (1:1, v/v). All solvents were evaporated from the mevalonolactone and the material was redissolved in sterile isotonic saline to give a final concentration of 10 μCi/ml.

Reference Compounds

Cholesterol (U.S.P., Nutritional Biochemicals Corp., Cleveland, OH) was recrystallized from ethanol. Colestipol hydrochloride (Colestid^R) was obtained as a gift from the Upjohn Co. (Kalamazoo, MI). 5α-Cholestane (Applied Science Laboratories, State College, PA) was used as an internal standard for the gas liquid chromatographic (GLC) separations of the neutral sterols and acidic steroids after preparations of trimethylsilylether derivatives.

3α,7α-Dihydroxy-12-keto-5β-cholanoic acid, used as internal standard for the bile acid analyses, was synthesized according to the Fieser and Rajagopalan procedure (8).

Thin Layer Chromatography (TLC)

The thin layer chromatographic separations were carried out exactly as previously described (9-11).

Gas Liquid Chromatography (GLC)

The methods and conditions of all GLC analyses have been described in detail (9-11). All analyses were carried out on a Hewlett-Packard 7610A gas chromatograph.

Methods for the Isolation and Quantitation of Neutral and Acidic Steroids from Feces

An aliquot of 0.5-1.0 g powdered fecal material from 2-day fecal pool was extracted with ethanol (80 ml) for 48 hr. Following extraction, fecal residues were allowed to stand for 15 min in 25 ml of ethanol containing saturated aqueous ammonium carbonate (2 ml) in order to elute bile acids bound to colestipol (1). The ethanolic ammonium carbonate solutions were combined with the 80-ml ethanol extracts in reservoir bottles and were evaporated to 20 ml under nitrogen. The samples then were refluxed for 1 hr in 1 N NaOH in 95% ethanol (20 ml) (mild hydrolysis). In 4 instances, the fecal residues were allowed to stand for 15 min in ethanol (25 ml) containing saturated aqueous ammonium carbonate (2 ml) to determine if any neutral sterols or plant sterols were bound to colestipol. These samples were evaporated, refluxed for 1 hr in 1 N NaOH in 95% ethanol (20 ml) and analyzed for fecal neutral sterols as described earlier.

The subsequent methods used for the isolation and quantitation of fecal neutral and acidic steroids have been described in detail (10,12,13). The material in each fraction was analyzed by GLC of the trimethylsilylether (TMSi) derivatives. 5α-Cholestane was used as an internal standard. The recovery of dietary β-sitosterol was used as an index to correct for losses of neutral sterols. Since the recovery was 90% or better, no corrections for losses of neutral steroids resulting from degradation in the intestinal tract were required.

Quantitation of the fecal bile acids was carried out using methods previously described (6). 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid served as recovery standard in each sample. Corrections for losses were made as required.

Methods for Determination of the Specific Activity and Concentration of Cholesterol in Plasma and Liver

These methods have been described in detail (6,7).

Radioactivity Measurements

All radioisotopic measurements were made using new scintillation glass vials on a Beckman LS-8000 liquid scintillation system (Beckman Instruments, Fullerton, CA).

The radioactivity in each sample was obtained after evaporation of the solvent by the addition of 10-12 ml of 2,5-bis[2-(2-tert-butylbenzoxazolyl)]-thiophene (BBOT), 4 g/ℓ in toluene. Each sample was corrected for background and quenching effects using appropriate blanks and standards.

Calculations and Statistics

Acidic steroid and neutral steroid output was determined by chromatographic techniques (combined TLC and GLC). Endogenous neutral sterol production was estimated as previously described (6,7,11,14-16).

Cholesterol absorption was determined as the difference between the dietary intake of cholesterol (determined chromatographically) and the unabsorbed dietary neutral steroid in the feces.

The unabsorbed neutral steroid (mg/day), cholesterol turnover (mg/day), and cholesterol balance (mg/day) were calculated as described below (6,7,15,16): Unabsorbed neutral sterols (mg/day) = daily fecal neutral sterol (mg/day) - daily endogenous neutral sterol (mg/day). Cholesterol turnover (mg/day) = daily fecal endogenous neutral steroid output (isotopic) (mg/day) + daily fecal acidic steroid output (chromatographic) (mg/day); cholesterol balance (mg/day) = cholesterol output - cholesterol input = daily fecal neutral sterols (mg/day) + daily fecal acidic steroids (mg/day) - daily cholesterol intake (mg/day). Student's t-test was used to determine significance.

RESULTS

Rats weighing ca. 250 g were injected with DL-[2-^{14}C] mevalonolactone to label their cholesterol pool. The rats were then fed the experimental diets for 14 days. The weight gain, daily food intakes and fecal outputs for all the rats were similar (Table I). Plasma cholesterol levels were not decreased by 14-day colestipol administration (average ± SD) [low-dose colestipol group (group 2) - 61.5 ± 9.0 mg/100 ml; high-dose colestipol group (group 3), 69.0 ± 12.1 mg/100 ml; control group (group 1), 65.3 ± 17.8 mg/100 ml]. Liver cholesterol levels were slightly decreased by colestipol administration (average ± SD) (group 2, 2.2 ± 0.1 mg/g; group 3, 2.0 ± 0.2 mg/g; group 1, 3.2 ± 0.8 mg/g). Biliary cholesterol levels were increased by colestipol administration (average ± SD), group 2, 0.13 ± 0.03 mg/ml; group 3, 0.17 ± 0.03 mg/ml; group 1, 0.08 mg/ml. Biliary bile acid concentrations were not significantly different (group 1, 8.93 ± 2.17 mg/ml, group 2, 9.51 ± 3.15 mg/ml; group 3, 7.82 ± 0.51 mg/ml).

The sp act of cholesterol in plasma and liver for the animals in each group on day 14 were similar, but not identical, and indicated that isotopic equilibrium of cholesterol had been achieved in these tissues during this short term metabolism study (Table II). The sp act of cholesterol in these tissues on day 14 were lowest in the high-dose colestipol group (group 3).

Sterol metabolism data for the rats fed the colestipol are summarized in Table III. Daily

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 (2)	238 ± 11 (230,246)	328 ± 6 (324,332)	23.2 ± 2.7 (18,27)	4.2 ± 0.2 (3.9,4.4)
2	0.42% Colestipol hydrochloride (4)	262 ± 3 (258,266)	333 ± 21 (310,358)	20.1 ± 3.0 (14,26)	3.8 ± 0.5 (3.0,4.6)
3	1.67% Colestipol hydrochloride (4)	249 ± 4 (244,254)	321 ± 14 (308,338)	21.5 ± 3.9 (11,26)	4.5 ± 0.6 (3.2,5.4)

[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 daily food intake from days 4-14 of the experiment (5 samples per animal, group 1, N=10, groups 2 and 3, N=20).

[d]Values represent the average daily fecal outputs (3 pools per animal) on days 9-14 of the experiment, group 1, N=6, groups 2 and 3, N=12.

TABLE II

Representative Specific Activities of Cholesterol in Liver and Plasma on Day 14[a]

Group no.		Animal[b]	Liver cholesterol sp act (dpm/mg)	Plasma cholesterol sp act (dpm/mg)
1	Control	A	1471	1903
		B	1833	2432
2	0.42% Colestipol hydrochloride	A	1518	1769
		B	909	976
		C	1299	1611
		D	1642	1545
3	1.67% Colestipol hydrochloride	A	621	570
		B	745	829
		C	699	688
		D	784	777

[a]Determined by combined TLC/GLC techniques and liquid scintillation counting on day 14 of the experiment.

[b]Represents the animals in each group.

TABLE III

Comparative Sterol Balance Data of Rats on Different Diets[a,b]

Group no.	Diet	Daily acidic steroid output (mg/day)	Daily neutral steroid output (mg/day)	Daily endogenous neutral sterol (mg/day)	Daily cholesterol absorption (mg/day)	Cholesterol turnover (mg/day)	Cholesterol intake (mg/day)	Cholesterol balance (mg/day)
1	Control	7.0 ± 2.8 (4.4,11.6)	15.4 ± 3.5 (11.7,21.4)	5.5 ± 2.4 (3.0, 9.6)	13.6 ± 2.6 (11.0,17.6)	12.6 ± 5.1 (8.0,21.6)	23.4 ± 1.4 (21.8,25.6)	-1.0 ± 6.7 [(-9.49),10.18]
2	0.42% Colestipol hydrochloride	12.2[c] ± 2.2 (9.3,16.0)	15.6 ± 2.9 (11.2,20.3)	6.2 ± 1.9 (3.6,10.1)	10.0 ± 3.0 (4.9,16.0)	18.4[c] ± 3.8 (13.9,24.2)	19.4 ± 3.3 (13.3,24.7)	8.4[c] ± 3.7 (3.4,16.4)
3	1.67% Colestipol hydrochloride	39.6[c,d] ± 5.2 (31.4,49.2)	24.5[c] ± 4.6 (16.3,31.8)	11.6[c] ± 4.0 (4.6,31.8)	7.7 ± 5.3 [(-2.0),16.5]	51.2[c,d] ± 7.4 (36.0,65.0)	20.6 ± 3.4 (13.3,24.7)	43.5[c,d] ± 7.7 (26.9,56.8)

[a]The calculations were made using relationships described in the experimental section.

[b]Values represent averages ± SD of 6 pools for control rats, 12 pools for 0.42% colestipol rats and 12 pools for 1.67% colestipol rats: numbers in parentheses represent the range.

[c]$P < 0.01$ compared to controls.

[d]$P < 0.01$ compared to group 2.

cholesterol intake was similar in both colestipol and control rats. Daily acidic steroid output was significantly increased by colestipol feeding, depending on the dose of colestipol administered (group 1, 7.0 ± 2.8 mg/day vs group 2, 12.2 ± 2.2 mg/day [$P < 0.005$]; group 3, 39.6 ± 5.2 mg/day [$P < 0.005$]).

Daily neutral steroid output of high-dose colestipol group (24.5 ± 4.6 mg/day) increased, compared to that of either control group (15.4 ± 3.5 mg/day) or low-dose colestipol group (15.6 ± 2.9 mg/day) ($P < 0.005$). Four fecal extracts were analyzed for the possible binding of fecal neutral sterols to colestipol itself. No neutral or plant sterols could be detected in these extracts. Similarly, daily endogenous neutral steroid output increased only in the high-dose colestipol group (group 3, 11.6 ± 4.0 mg/day vs control, 5.5 ± 2.4 mg/day [$P < 0.005$]; group 2, 6.2 ± 1.9 mg/day). On the other hand, daily dietary cholesterol absorption was reduced by colestipol feeding, depending on the dose of colestipol administered (group 2, 10.0 ± 3.0 mg/day and group 3, 7.7 ± 5.3 mg/day vs group 1, 13.6 ± 2.6 mg/day [$P < 0.025$]). Moderate increases in cholesterol turnover for low-dose colestipol-fed rats were observed (from 12.6 ± 5.1 mg/day to 18.4 ± 3.8 mg/day), group 1 vs group 2 ($P < 0.01$).

Similarly, moderate increases in cholesterol balance for low-dose colestipol-fed rats was observed (from -1.0 ± 6.7 mg/day to 8.4 ± 3.7 mg/day), group 1 vs group 2 ($P < 0.005$). In contrast, large increases in cholesterol turnover and cholesterol balance for high-dose colestipol-fed rats were observed (from 12.6 ± 5.1 mg/day to 51.2 ± 7.4 mg/day, and from 1.0 ± 6.7 mg/day to 43.5 ± 7.7 mg/day, respectively), group 1 vs group 3 ($P < 0.005$).

DISCUSSION

This study reports the effects of 0.42% colestipol (equivalent 500 mg/kg/day) and 1.67% colestipol (equivalent 2000 mg/kg/day) administration on: (a) bile acid synthesis; (b) cholesterol absorption and (c) cholesterol balance.

Colestipol binds bile acids and prevents their reabsorption in the intestine (1). This results in a greatly increased fecal excretion of bile acids. As expected, the daily fecal acidic steroid output was greatly increased by colestipol administration (0.42% colestipol administration, 1.7-fold; 1.67% colestipol administration, 5.7-fold, compared to control group). This suggests that bile acid synthesis from cholesterol in the liver was remarkably accentuated

by colestipol administration. It has been reported that the maximal synthesis rate for taurocholic acid in a bile fistula rat is 15 mg/100 g rat/day (16). Thus, a fecal bile acid excretion rate of 39.6 mg/rat/day (average wt 321 g) is within the limits for synthesis by the animal. It is well known that cholesterol is absorbed from the intestine by micelle formation with bile acids (17). In this experiment, intestinal absorption of exogenous (dietary) cholesterol was suppressed and the daily endogenous neutral steroid output was increased by colestipol administration. These findings suggest that intestinal absorption of both exogenous (dietary) and endogenous cholesterol was decreased by colestipol administration because there were not enough bile acids in the intestine to form micelles indispensable for the intestinal cholesterol absorption. No neutral sterols were found bound to colestipol suggesting that indeed the lack of bile acids was responsible for the decreased cholesterol absorption. Cholesterol turnover which reflects the daily fecal excretion of the materials related to endogenous cholesterol was increased dose-dependently, accompanied by increased daily acidic steroid output and increased daily endogenous neutral steroid output. Consequently, colestipol administration caused marked increases (dose-dependent) in cholesterol balance, a measurement which reflects relative cholesterol synthesis.

In summary, colestipol caused a greatly increased fecal excretion of bile acids and consequently, the catabolism of cholesterol was remarkably stimulated. Meanwhile, intestinal absorption of both exogenous and endogenous cholesterol was decreased by colestipol administration.

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Effect of Diet on Choline Phosphotransferase, Phosphatidylethanolamine Methyltransferase and Phosphatidyldimethylethanolamine Methyltransferase in Liver Microsomes

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ABSTRACT

Phosphatidylcholine (PC) biosynthesis has been investigated in female rats fed a liquid amino acid, choline-methionine-free diet by assaying in liver microsomes the specific and total activities of choline phosphotransferase, phosphatidyldimethylethanolamine methyltransferase and phosphatidylethanolamine methyltransferase. There was a significant decrease in the specific activity (sp act) of choline phosphotransferase in the liver of rats fed a choline-methionine-free diet. The dietary omission of methionine for 2 wk resulted in a significant decrease in the sp act of choline phosphotransferase. The dietary omission of choline, methionine, B_{12}, folic acid and the addition of a methyl group acceptor, guanidoacetic acid, decreased further the sp act of choline phosphotransferase. The phosphatidylethanolamine methyltransferase sp act increased with the dietary omission of choline and methionine. The dietary omission of choline, methionine, B_{12}, folic acid and the addition of a methyl group acceptor, guanidoacetic acid, resulted in a decrease in the sp act of phosphatidyldimethylethanolamine methyltransferase and an increase in phosphatidylethanolamine methyltransferase. The dietary omission of choline, methionine, B_{12}, folic acid and the addition of a methylation inhibitor, 2-amino-2-methyl-1-propanol, did not result in a significant decrease in the sp act of choline phosphotransferase; however, it did significantly decrease the sp act of phosphatidylethanolamine methyltransferase. The addition of dietary methionine with the inhibitor resulted in a significant decrease in the sp act of the choline phosphotransferase and phosphatidylethanolamine methyltransferase when compared to control and/or when compared to deficient with or without inhibitor. The dietary supply of methionine, as a source of choline, did affect the activity of the enzymes that synthesize PC. The ratio of the substrate, S-adenosylmethionine, and the inhibitory product, S-adenosylhomocysteine, affected the enzymatic activity of phosphatidylethanolamine methyltransferase. It is suggested that the concentrations of these 2 compounds may be important in regulating the methylation of phosphatidylethanolamine in the liver cell.

INTRODUCTION

Phosphatidylcholine (PC) biosynthesis in liver microsomes is known to occur by 2 major different pathways. The Kennedy (1) pathway involves choline phosphotransferase which catalyzes the following reaction: cytidine diphosphocholine + 1,2-diacylglycerol to form PC + CMP. The Bremer-Greenberg (2) pathway involves phosphatidylethanolamine methyltransferase which catalyzes the following reaction: phosphatidylethanolamine + S-adenosylmethionine with progressive methylation to form PC. In this report, the effect of the dietary omission of choline and methionine on the enzymatic activities of choline phosphotransferase, phosphatidylethanolamine methyltransferase and phosphatidyldimethylethanolamine methyltransferase has been determined in liver microsomes of female rats fed a purified liquid amino acid diet.

MATERIALS AND METHODS

Female albino rats of the Sprague-Dawley strain (60-80 days old, 140-180 g) were used in all experiments. They were kept in individual wire-bottomed cages with a dark period from 8:00 p.m. to 8:00 a.m. The animals were fed the amino acid liquid diet (3) without choline or methionine for 1, 2, 3, 7 and 14 days. Controls were fed the amino acid liquid diet containing choline and methionine. The effects of further methyl group depletion were investigated by the dietary addition of a methyl group acceptor, guanidoacetic acid (4). The liver converts guanidoacetic acid to creatine which is excreted in the urine. Additional experiments were set up to investigate the effects of a methylation inhibitor, 2-amino-2-methyl-1-propanol (5,6).

The components of liquid diet expressed as g/100 ml were as follows: essential amino acids mixture, 2.307; nonessential amino acids, 2.422; salt mixture (Hegsted IV), 1; vitamin mixture, 1.250; Mazola corn oil, 1.250; cod liver oil, 0.375; Pastene olive oil, 3.000; sodium carrageenate (Viscarin), 0.400; sucrose, 8.75;

distilled water, up to 100 ml. The composition of the essential amino acid mixture in g/100 g was as follows: L-lysine HCl, 13.434; L-arginine HCl, 8.125; DL-tryptophan, 2.166; DL-phenylalanine, 9.750; DL-leucine, 17.334; DL-isoleucine, 10.834; DL-valine, 15.167; L-histidine HCl, 5.850; DL-methionine, 6.500; DL-threonine, 10.834. The composition of the nonessential amino acid mixture, g/100 g, was as follows: L-glutamic acid, 20.639; DL-serine, 5.159; glycine, 7.223; DL-tyrosine, 28.895; L-cystine, 2.063; L-proline, 9.287; L-asparagine monohydrate, 14.334; DL-alanine, 12.383. The composition of the vitamin mixture in mg/100 g was as follows: thiamine HCl, 5; riboflavin, 10; pyridoxine HCl, 5; calcium pantothenate, 40; nicotinamide, 30; choline chloride, 2500; biotin, 0.2; folic acid, 2; inositol, 200; 2-methyl-1,4-naphthoquinone, 2; vitamin B_{12}, 0.2; *p*-aminobenzoic acid, 100; sucrose, 97,610. The diet constituents were purchased from General Biochemicals (Teklad Test Diets, Madison, WI).

At the end of the dietary regime, the rats were killed by decapitation, the livers removed, rinsed with cold water, blotted and homogenized in ice-cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. The microsomal fraction was isolated by differential centrifugation (7). The nuclear and mitochondrial fractions were separated from the homogenate by centrifuging for 10 min at 14,500 g. The supernatant solution was centrifuged at 78,450 g for 60 min to sediment the microsomal pellet. Protein was determined by a modified Biuret method (8).

Choline Phosphotransferase Assay

The assay of the reaction catalyzed by the enzyme CDP-choline: 1,2-diacylglycerol choline phosphotransferase (EC 2.7.8.2) was done by the Kennedy method (1). The materials used were cytidine diphosphate-[1,2-^{14}C]-choline (ICN Tracerlab Chemical and Isotope Division, Irvine, CA) and Tween-20 (Sigma Chemical Co., St. Louis, MO). Diacylglycerol was prepared from egg lecithin by the Gurr et al. method (9) and purified by the Barron and Hannahan chromatographic method (10). Each reaction mixture contained 60 μmol 1,2-diacylglycerol emulsified in 0.1 ml of 1% Tween-20, 10 μmol $MgCl_2$, 0.5 μmol CDP-[1,2-^{14}C] choline (sp act, 4 x 10^5 cpm/μmol), and 10 mg microsomal protein. The final volume of the reaction mixture was 1.3 ml. The reaction time was 6 min. The reaction was terminated by the addition of 3.0 ml 95% ethanol and the product was isolated by repeated extraction with ethanol and chloroform (1).

Phosphatidyldimethylethanolamine Methyltransferase Assay

The assay of the last step in the methylation of phosphatidylethanolamine to PC, catalyzed by phosphatidyldimethylethanolamine methyltransferase, was performed using the Rehbinder and Greenberg method (11). The reaction mixture contained 1.75 mM dipalmitoyl phosphatidyldimethylethanolamine (Sigma Chem. Co.), 6.3 mM sodium deoxycholate, 0.3 M Tris-HCl buffer, pH 8.6, 0.35 mM S-adenosyl-L-[methyl-^{14}C] methionine (0.1 mCi/mMol, New England Nuclear) and microsomes (1-2 mg) in a final volume of 1.15 ml. The assay was initiated by the addition of microsomes. Reaction time was 15 min at 37 C. The reaction was terminated with 0.20 ml HCl and the product was isolated by extraction with n-butanol (11).

Phosphatidylethanolamine Methyltransferase Assay

The rate-limiting step in the methylation pathway was measured by modified method of Bremer and Greenberg (12). The reaction mixture contained 2 mg egg PC (Sigma Chemical Co., St. Louis, MO), 0.9 mM sodium deoxycholate, 0.29 M Tris-HCl buffer, pH 8.6, 0.2 mM S-adenosyl-L-[methyl-^{14}C]methionine (0.1 mCi/mMol, New England Nuclear, Boston, MA) and microsomes (1-2 mg protein) in a final volume of 1.4 ml. The assay was initiated by adding microsomes, reaction time was 15 min at 37 C, and the reaction was terminated with 0.15 ml HCl. The ^{14}C-labeled reaction product, PC, was extracted according to the Bligh and Dyer procedure (13).

RESULTS

The enzymatic activities of choline phosphotransferase, phosphatidyldimethylethanolamine methyltransferase and phosphatidylethanolamine methyltransferase were linear with time and concentration of enzyme. The data are not presented but are similar to those reported previously (14).

The effect of dietary omission of choline and methionine for 1, 2, 3, 7 and 14 days on sp act of choline phosphotransferase, phosphatidyldimethylethanolamine methyltransferase and phosphatidylethanolamine methyltransferase (nmol PC formed/min/mg protein) and total liver microsomal activity (nmol PC formed/min) are given in Table I. There was a significant decrease in the sp act of the choline phosphotransferase and phosphatidyldimethylethanolamine methyltransferase with the omission of choline and methionine for day one. There was a significant decrease in specific

TABLE I

Effect of Dietary Omission of Choline and Methionine on Choline Phosphotransferase, Phosphatidyldimethylethanolamine Methyltransferase and Phosphatidylethanolamine Methyltransferase Activities in Liver Microsomes from Female Rats Fed Purified Amino Acids Liquid Diet

Time of diet	No. of animals	Specific activity (nmol/min/mg protein) x 10		
		Choline phosphotransferase	Phosphatidyl-dimethylethanolamine methyltransferase	Phosphatidyl-ethanolamine methyltransferase
Days				
0 (Controls)	6	5.33 ± 1.15[a] (86.5 ± 5.7)	13.64 ± 1.76 (239.4 ± 54.5)	0.93 ± 0.19 (15.9 ± 2.1)
1	3	3.32 ± 0.82[b] (90.1 ± 22.1)	10.17 ± 0.45[c] (268.8 ± 17.3)	0.83 ± 0.04 (21.8 ± 2.5)[c]
2	4	2.09 ± 0.36[c] (44.2 ± 2.6)[d]	12.99 ± 1.41 (276.0 ± 10.9)	1.23 ± 0.22[b] (26.0 ± 2.7)[d]
3	3	3.74 ± 1.28[b] (78.2 ± 16.7)	8.88 ± 1.42[c] (188.7 ± 18.7)	0.98 ± 0.10 (21.0 ± 1.7)[c]
7	3	3.57 ± 0.31[b] (84.1 ± 11.4)	11.87 ± 2.09 (276.7 ± 18.0)	1.29 ± 0.32[b] (29.8 ± 2.2)[d]
14	6	3.21 ± 0.63[d] (55.6 ± 13.0)[d]	11.29 ± 3.24 (198.8 ± 66.3)	1.33 ± 0.28[c] (23.7 ± 3.9)[d]

[a]± Values are standard deviations. The values in parentheses indicate the total liver microsomal activity (nmol/min). The test of significance was applied between the mean value of controls receiving 31.25 mg choline and 0.15 g DL-methionine/100 ml of diet and the deficient animals.

[b]$P < 0.05$.

[c]$P < 0.005$.

[d]$P < 0.001$.

and total microsomal liver activities for the choline phosphotransferase in the animals fed the diet with the omission of choline and methionine for 2 and 14 days. There was also a significant decrease in the sp act of the choline phosphotransferase with the dietary omission for 7 days. The sp act of the phosphatidyldimethylethanolamine methyltransferase was significantly decreased by the dietary omission of choline and methionine for 1 and 3 days. A significant increase was observed in the total liver microsomal activity of the phosphatidylethanolamine methyltransferase with the dietary omission for 1, 2, 3 and 14 days. There was a significant increase in the sp act of phosphatidylethanolamine methyltransferase with the dietary omission of choline and methionine at 2, 7 and 14 days.

The data for additional rats that were fed the diet with the omission of choline and/or methionine for 2 wk are given in Table II. There were no significant changes in the biosynthesis of choline as seen in the sp act or total liver activities of choline phosphotransferase, phosphatidyldimethylethanolamine transferase and phosphatidylethanolamine methyltransferase if only choline was omitted and rats received dietary methionine for 2 wk. However, if animals were fed a diet deficient in methionine, a significant decrease in the total liver microsomal choline phosphotransferase activity and a significant increase in the sp act of phosphatidylethanolamine methyltransferase occurred. The dietary omission of both choline and methionine for 2 wks was required to depress the sp act and total liver microsomal activity of the choline phosphotransferase whereas phosphatidylethanolamine methyltransferase was conversely stimulated.

The effects of the dietary omission of choline, methionine, B_{12} and folic acid and the addition of a methyl group acceptor, 1% guanidoacetic acid, for 7 and 14 days on the enzymes involved in PC biosynthesis in liver microsomes are given in Table III. There was a significant decrease in the total liver microsomal activity of the choline phosphotransferase with the dietary omission of choline, methionine, B_{12} and folic acid at 7 and 14 days and a significant decrease in the sp act of liver microsomes at 14 days when compared to controls. There was a significant increase in the sp act and total microsomal activity of phosphatidylethanolamine methyltransferase with the dietary omission of choline, methionine, B_{12} and folic

TABLE II

Effect of Dietary Omission of Choline and Methionine on Choline Phosphotransferase, Phosphatidyldimethylethanolamine Methyltransferase and Phosphatidylethanolamine Methyltransferase Activities in Liver Microsomes from Female Rats Fed Purified Amino Acids Liquid Diet for Two Weeks

Dietary regimen	No. of animals	Specific activities (nmol/min/mg protein) x 10		
		Choline phosphotransferase	Phosphatidyl-dimethylethanolamine methyltransferase	Phosphatidyl-ethanolamine methyltransferase
Control	6	5.33 ± 1.15[a] (86.5 ± 5.7)	13.64 ± 1.76 (239.4 ± 54.5)	0.93 ± 0.19 (15.9 ± 2.1)
Deficient choline and methionine	6	3.21 ± 0.63[d] (55.2 ± 12.7)[d]	11.29 ± 3.24 (198.8 ± 66.3)	1.33 ± 0.28[c] (23.7 ± 3.9)[d]
Deficient methionine	6	5.16 ± 1.02[b] (73.1 ± 12.2)	12.17 ± 2.70 (188.8 ± 50.1)	1.31 ± 0.38[b] (18.4 ± 4.1)
Deficient choline	6	4.83 ± 2.33 (87.6 ± 27.3)	12.51 ± 2.53 (232.0 ± 25.4)	0.79 ± 0.16 (14.6 ± 1.3)

[a]± Values are standard deviations. The values in parentheses indicate the total liver microsomal activity (nmol/min). The test of significance was applied between the mean values of controls and deficient animals.

[b]$P < 0.05$.

[c]$P < 0.005$.

[d]$P < 0.001$.

TABLE III

Effect of Dietary Omission of Choline, Methionine, B_{12}, and Folic Acid and the Addition of a Methyl Group Acceptor, 1% Guanidoacetic Acid, on Choline Phosphotransferase, Phosphatidyldimethylethanolamine Methyltransferase and Phosphatidylethanolamine Methyltransferase Activities in Liver Microsomes from Female Rats Fed Purified Amino Acids Liquid Diet

Time of diet	No. of animals	Dietary regimen	Specific activity (nmol/min/mg protein) x 10		
			Choline phosphotransferase	Phosphatidyl-dimethylethanolamine methyltransferase	Phosphatidyl-ethanolamine methyltransferase
Days					
7	6	Control	3.29 ± 0.74[a] (83.4 ± 13.3)	10.54 ± 1.33 (269.4 ± 34.2)	0.99 ± 0.12 (25.2 ± 2.6)
	6	Deficient	2.69 ± 0.42 (59.2 ± 3.0)[c]	11.77 ± 2.7 (256.7 ± 31.0)	1.46 ± 0.18[c] (32.2 ± 3.4)[d]
	6	Deficient + 1% guanidoacetic acid	1.33 ± 0.26[d,f] (22.0 ± 3.3)[d,f]	11.49 ± 1.34 (192.6 ± 15.6)[d,f]	1.71 ± 0.11[d,h] (28.7 ± 1.6)[c]
14	5	Control	5.33 ± 1.15 (86.5 ± 5.73)	13.64 ± 1.76 (239.4 ± 54.5)	0.93 ± 0.19 (15.9 ± 2.1)
	5	Deficient	3.95 ± 0.54[b] (70.0 ± 3.20)[d]	16.63 ± 2.12[b] (293.8 ± 26.5)	2.21 ± 0.21[d] (41.2 ± 3.2)[d]
	5	Deficient + 1% guanidoacetic acid	0.74 ± 0.22[d,f] (12.7 ± 2.3)[d,f]	5.16 ± 1.29[d,f] (91.3 ± 22.7)[d,f]	1.96 ± 0.25[d] (35.5 ± 3.9)[d,i]

[a]± Values are standard deviations. The values in parentheses indicate the total liver microsomal activity (nmol/min). The test of significance was applied between the mean values of controls receiving 31.25 mg choline, 0.15 g DL-methionine, B_{12} and folic acid/100 ml of diet and the deficient animals.

[b]$P < 0.05$.

[c]$P < 0.005$.

[d]$P < 0.001$,

[e]$P < 0.01$

The test of significance was applied between the mean value of deficient and the animals deficient + 1% guanidoacetic acid.

[f]$P < 0.001$.

[g]$P < 0.005$.

[h]$P < 0.01$.

[i]$P < 0.05$.

acid for 7 and 14 days when compared to controls. There was a significant decrease in the sp act and total liver microsomal activity of choline phosphotransferase when the methyl group acceptor, 1% guanidoacetic acid, was added to the deficient diet for 7 and 14 days when compared to controls. The addition of guanidoacetic acid further depleted the animals of methionine and resulted in a significant decrease in the sp act of the choline phosphotransferase when compared to the 7 and 14-day deficient and/or control animals.

A significant decrease in the total liver microsomal activity of phosphatidyldimethylethanolamine methyltransferase was observed in animals receiving guanidoacetic acid for 7 days, when compared to controls and/or deficient animals. The addition of guanidoacetic acid for 14 days resulted in a significant decrease in both specific and total microsomal activities of phosphatidyldimethylethanolamine methyltransferase when compared to controls and/or deficient animals.

There was a significant increase in the sp act of phosphatidylethanolamine methyltransferase in the deficient animals for 7 and 14 days when compared to controls. The animals receiving guanidoacetic acid for 7 and 14 days showed a significant increase in the total liver microsomal activity when compared to the deficient animals.

The effects of dietary omission of choline, methionine, B_{12}, folic acid and the addition of a methylation inhibitor, 2-amino-2-methyl-1-propanol (0.33%), for 7 days on the enzymes involved in PC biosynthesis are given in Table IV. A significant decrease occurred in the total liver microsomal activity of choline phosphotransferase when compared with control animals. There was also a significant decrease in the sp act and total liver microsomal activity of the phosphatidylethanolamine methyltransferase. There was a significant increase in the sp act of phosphatidyldimethylethanolamine methyltransferase when compared to controls. Thus, additional experiments were set up to measure the effects of the inhibitor in the presence of dietary methionine. The methylation inhibitor in the presence of dietary methionine resulted in a significant decrease in the sp

TABLE IV

Effect of Dietary Omission of Methionine, Choline, B_{12}, Folic Acid and the Addition of a Methylation Inhibitor, 2-Amino-2-methyl-1-propanol (0.33%), on Choline Phosphotransferase, Phosphatidyldimethylethanolamine Methyltransferase and Phosphatidylethanolamine Methyltransferase Activities in Liver Microsomes from Female Rats Fed Purified Amino Acids Liquid Diet for Seven Days

Dietary regimen	No. of animals	Specific activity (nmol/min/mg protein) x 10		
		Choline phosphotransferase	Phosphatidyl-dimethylethanolamine methyltransferase	Phosphatidyl-ethanolamine methyltransferase
Control	6	3.29 ± 0.74[a] (83.4 ± 13.3)	10.54 ± 1.33 (269.4 ± 34.3)	0.99 ± 0.12 (25.2 ± 2.6)
Deficient	6	2.69 ± 0.42 (59.2 ± 3.0)	11.77 ± 2.70 (256.7 ± 31.0)	1.46 ± 0.12 (32.2 ± 3.4)
Deficient + inhibitor	4	3.22 ± 0.70 (63.1 ± 3.2)[c]	14.18 ± 1.54[b] (282.3 ± 28.7)	0.71 ± 0.17[c,e] (13.9 ± 0.7)[b,e]
Deficient + methionine + inhibitor	3	1.43 ± 0.3[b,e] (63.4 ± 6.1)[h,d]	9.30 ± 2.37[h] (345.4 ± 52.7)[d,f,i]	0.56 ± 0.09[b,e] (21.3 ± 3.0)[d,e,g]

[a]± Values are standard deviations. The values in parentheses indicate the total liver microsomal activity (nmol/min). The test of significance was applied between controls receiving methionine, choline, B_{12} and folic acid and the deficient animals receiving the 2-amino-2-methyl-1-propanol.

[b]$P < 0.001$.

[c]$P < 0.01$.

[d]$P < 0.05$.

The test of significance was applied between deficient animals and those receiving the inhibitor.

[e]$P < 0.001$.

[f]$P < 0.005$.

The test of significance was applied between deficient animals receiving inhibitor and those receiving methionine plus inhibitor.

[g]$P < 0.001$.

[h]$P < 0.005$.

[i]$P < 0.05$.

act of the choline phosphotransferase when compared to controls and/or deficient with or without inhibitor. There was a significant reduction of the specific and total liver microsomal activities of the phosphatidylethanolamine methyltransferase when compared to controls and/or deficient animals. There was a significant increase in total liver microsomal activity of phosphatidyldimethylethanolamine methyltransferase when compared to controls and/or deficient animals with or without inhibitor.

The inhibition of phosphatidylethanolamine methyltransferase by various concentrations of AdoMet/AdoHcy is given in Figure 1. The reaction conditions were similar to those described in the Materials and Methods section for the enzyme assay, except that various concentrations of nonradioactive S-adenosylmethionine and S-adenosylhomocysteine were added to the reaction mixture. Apparently, there is inhibition of phosphatidylethanolamine methyltransferase activity at all ratios of AdoMet/AdoHcy.

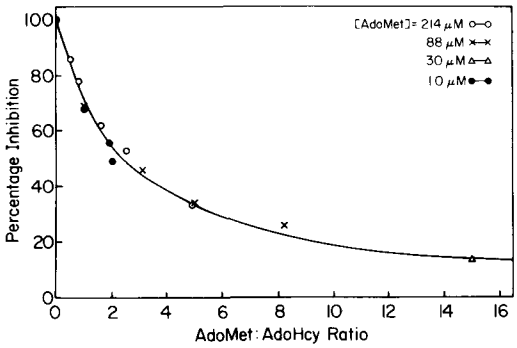

FIG. 1. Inhibition of rat liver phosphatidylethanolamine methyltransferase specific activity. Points are means of duplicate determinations. Reaction conditions were as described in Materials and Methods with varying concentrations of S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) as listed.

DISCUSSION

Several commercially available choline-free diets which most investigators have used to produce choline deficiency have present proteins which contain large amounts of methionine. duVigneaud et al. (15-17) were the first to show in rats fed a choline-methionine-free diet that labeled methionine is converted to choline. These investigators demonstrated in rats fed a choline-methionine-free diet that 89% of the choline was derived from dietary deuteromethionine. Thus, in producing a choline deficiency in animals, the methionine content of the food used should be calculated. The diets used by Artom and Cornatzer (18) to produce choline deficiency in 1 or 2 wk were free of choline and contained 5% casein. This 5% casein diet contained 1.29 mg methionine/g food and represented one of the diets lowest in methionine. In addition, these investigators would supplement this 5% casein diet with guanidoacetic acid, a methyl acceptor, to further deplete the animal of available methyl groups (19,20). Borsook and Dubnoff (4) were the first to show in rat liver that methionine will methylate guanidoacetic acid to form creatine, which is excreted in the urine. Stetten and Grail (21) demonstrated that guanidoacetic acid feeding decreased the PC content of the liver.

Cornatzer and Walser (22) demonstrated that in rats fed a 5% casein, choline-free diet, there is a significant decrease in total liver PC and in mitochondrial PC (μg/g liver). This decrease was much more prevalent in mitochondria and microsomes when expressed as μg lipid P/mg protein than per g liver (23). Haines and Rose (24) have shown that, in choline deficiency for 3 days in the rat, the PC content of the liver decreased. It is well established that the dietary omission of the lipotropic agents, choline and/or methionine results in a fatty liver. These studies were set up to investigate PC biosynthesis by the 2 different enzymatic pathways in microsomes prepared from rats fed a diet free of choline and methionine. Additional animals were fed similar deficient diets in which B_{12} and folic acid were also omitted to prevent the conversion of cellular homocysteine back to methionine (25). The methyl acceptor, guanidoacetic acid, was also included in the diet to further deplete the liver cells of methionine and decrease the concentration of choline. The effects of feeding a methylation inhibitor, 2-amino-2-methyl-1-propanol, were also studied. This compound inhibits the formation of choline and the conversion of phosphatidylethanolamine to PC (5).

It is well established that there is a tissue pool of choline and methionine. These pools are influenced by dietary intake, cellular synthesis and metabolism. The cellular synthesis of methionine depends on dietary folic acid and B_{12}. The cellular pool of methionine is in equilibrium with tissue proteins. The fluctuations in the sp act reported in Table I of the choline phosphotransferase and phosphatidyldimethylethanolamine methyltransferase involved in PC biosynthesis during the early choline-methionine deficiency may represent some type of body adjustment in the pool of

these compounds. Yamamoto et al. (26) reported there are different stages of PC biosynthesis in rats made choline-deficient for 4, 12 and 20 days; first, a decrease in PC biosynthesis is observed, followed by an increased incorporation in [^{14}C-methyl] methionine into PC. Turkki and Silvestre (27) observed that the concentration of total phospholipid-P (μg/g liver) decreased in rats fed a choline-deficient diet for 1 day, increased on the second and third days and subsequently decreased at 7 and 14 days when compared to controls.

It is apparent from the data that the dietary omission of choline and methionine for 2 wk decreased significantly the sp act of the choline phosphotransferase and increased the sp act of the phosphatidylethanolamine methyltransferase. The data show that the dietary omission of only methionine for 2 wk plays a more important role than the dietary omission of choline in regulating the activity of the enzymes involved in PC biosynthesis. This observation shows how important the methyl group of methionine is for the biosynthesis of choline and further substantiates the work of duVigneaud et al. (15-17). Wise and Elwyn (28) have estimated that the methylation pathway can provide 13 μmol choline/day/g of liver (4 mg/day/3 g liver) or equivalent to the normal dietary intake of choline for the rat.

As S-adenosylmethionine donates a methyl group, the product, S-adenosylhomocysteine, is formed. S-adenosylhomocysteine is converted to homocysteine plus adenosine by S-adenosylhomocysteine hydrolase. Two different enzymatic pathways are known to convert homocysteine back to methionine in the liver using folic acid and B_{12} as cofactors (29,30). One enzyme, N-methyltetrahydrofolate-homocysteine methyltransferase, uses B_{12} as cofactor (31). The second enzyme, betaine-homocysteine methyltransferase, uses a methyl group from betaine, a metabolic product of choline (32). A significicant decrease resulted in the sp act of choline phosphotransferase and the sp act of phosphatidylethanolamine methyltransferase increased with the dietary omission of choline, methionine, B_{12} and folic acid for 14 days when compared to controls.

The dietary omission of choline, methionine, B_{12}, folic acid and the addition of a methyl acceptor, guanidoacetic acid, for 7 and 14 days significantly decreased the specific and total liver microsomal activities of choline phosphotransferase. This exaggerated further the effects of the dietary omission of methionine.

Wells (5) was the first to observe that 2-amino-2-methyl-1-propanol inhibits choline formation and the methylation of phosphatidylethanolamine to from PC. In his experiments with the inhibitor, dietary methionine was always present. It is apparent from the data of this study that 2-amino-2-methyl-1-propanol inhibited significantly the biosynthesis of PC by the choline phosphotransferase and the phosphatidylethanolamine methyltransferase pathways.

Other investigators have observed an increased conversion of phosphatidylethanolamine to PC in rats fed a choline-free diet (33-35). However, the methionine content was not low in the diets fed by the investigators. Haines and Rose (24) have observed an increase in the liver concentration of phosphatidylethanolamine in rats fed a choline-free diet for 3 days. Tinoco et al. (36) have demonstrated that the phosphatidylethanolamine concentration increases and the PC concentration decreases in liver microsomes in choline deficiency.

The 2 methyltransferases, phosphatidyldimethylethanolamine methyltransferase and phosphatidylethanolamine methyltransferase, may be under a different type of control or the in vitro assay may reflect a variance in the presence of endogenous substrate for each. Phosphatidylethanolamine is present in large quantities in vivo whereas the concentration of phosphatidyldimethylethanolamine may be low.

The increase in the enzymatic activity of the phosphatidylethanolamine methyltransferase that is observed in the animals fed a choline-methionine-free diet in this study could be the result of the increased phosphatidylethanolamine content or it could have been caused by a change in the ratio of the concentration of S-adenosylmethionine (AdoMet) to S-adenosylhomocysteine (AdoHcy) in the cell. AdoMet serves as the methyl donor in a host of biological reactions. AdoHcy, one of the products of all transmethylation reactions involving AdoMet, acts as a competitive inhibitor with respect to this substrate. Gibson et al. (37) presented the first evidence that AdoHcy was inhibtory to phosphatidylethanolamine methyltransferase. Kaneshiro and Law (38) have shown that AdoHcy at 4 μM inhibited by 50% the methylase of *Agrobacterium tumefacrens.* Many of the methyltransferases including those that catalyze the methylation of phosphatidylethanolamine (39) have a higher affinity for AdoHcy than AdoMet. Other investigators (40-42) have suggested that the availability of substrate, AdoMet, for the methyltransferases is probably a regulator of transmethylation. Thus, the ratio of AdoMet-to-AdoHcy may be bioregulatory in certain nutritional stress condi-

tions. Finkelstein and Harris (43) have shown that the synthesis of AdoHcy is decreased in rats fed a low-protein diet. Eloranta (44) has demonstrated that intraperitoneal injection of methionine resulted in an increased concentration of both AdoMet and AdoHcy in the liver tissue. He concluded that the concentration of these compounds is dependent on the methionine supply. Åkesson (45) has demonstrated that the rate of methylation is influenced by the concentration of methionine and ethanolamine in hepatocytes. He concluded that the concentration of methionine and phosphatidylethanolamine in the cell will affect phospholipid methylation. Thus, in the rats fed a choline-methionine-free diet in the experiments we have reported, the concentration of AdoMet and AdoHcy may be changed and could affect the enzymatic activity of the phosphatidylethanolamine methyltransferase.

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Distribution of the Phosphatidate Phosphohydrolase Activity in the Lamellar Body and Lysosomal Fractions of Lung Tissue

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ABSTRACT

Phosphatidate phosphohydrolase (PAPase) activity was measured in lamellar bodies purified from porcine lung tissue. After repeated freeze-thawing, only a negligible amount of PAPase activity was released into the soluble fractions, whereas there was release of 2 lysosomal marker enzyme activities, glucosaminidase and β-galactosidase into the soluble fraction. In addition, a lysosomal-enriched fraction was prepared from adult rat lung tissue by prior treatment of the rats with Triton WR 1339. Treatment with Triton WR 1339 resulted in the significant shift of the activities of the lysosomal marker enzymes, glucosaminidase and β-galactosidase, to less dense subcellular fractions. The highest specific activity of PAPase was found in a subcellular fraction which had a density that was intermediate between that of the mitochondrial and microsomal fractions and the distribution of PAPase activity was not affected by the prior treatment of the rats with Triton WR 1339.

INTRODUCTION

Based on the results obtained in previous investigations, we concluded that the enzyme phosphatidate phosphohydrolase (PAPase [EC 3.1.3.4]) was associated with lamellar bodies isolated from adult porcine lung tissue. In these studies, we observed that the specific activity (sp act) of PAPase in lamellar bodies was ca. 2 times that of the enzyme in lung tissue microsomes (1). Subsequently, we found that PAPase activity was closely associated with lamellar bodies isolated from human amniotic fluid (2), and with purified surfactant obtained by pulmonary lavage (3). Meban (4), employing histochemical techniques, demonstrated that PAPase activity was closely associated with the osmiophilic granules of the type II pneumonocytes. On the other hand, Garcia et al. (5) concluded that the PAPase activity which is associated with lamellar bodies isolated from rat lung tissue can be accounted for by microsomal contamination. However, employing the technique described by McMurray (6), we reassessed the origin of the PAPase activity present in porcine lamellar bodies and demonstrated that the activity of this enzyme was not attributable to microsomal contamination (7).

The subcellular distribution of PAPase in rat liver tissue has been described (8,9). In these studies, a significant amount of the PAPase activity was found to be associated with the subcellular fraction containing the greatest amount of lysosomal enzyme activities. The PAPase activity could be released from rat liver lysosomes by the procedure of repeated freezing and thawing (8). Mavis et al. (10) suggested that the activity of PAPase which is associated with lamellar bodies may result from contamination of the lamellar body preparation with lysosomes. The purpose of this investigation was to evaluate the contribution of lysosomes to the PAPase activity associated with purified porcine lamellar bodies and to assess the PAPase activity in a purified lysosomal fraction prepared from rat lung tissue.

MATERIALS AND METHODS

Mitochondrial, microsomal and lamellar body fractions were prepared from adult porcine lung tissue employing techniques previously described (7). Each fraction was resuspended in 0.25 M sucrose. It was found that in these subcellular preparations more than 85% of the cytochrome C oxidase (EC 1.9.3.1) and NADPH cytochrome C reductase (EC 1.6.2.4) activity was associated with the mitochondrial and microsomal fractions, respectively. The lamellar body fraction contained ca. 8 mg phospholipid/mg protein. We analyzed the phosphatidylcholine (PC) of this fraction employing the procedure described by Mason et al. (11), and fatty acid composition employing gas chromatography and found that it was comprised of ca. 70-80% 1,2 dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoyl-phosphatidylcholine). Based on the NADPH cytochrome C reductase (EC 1.6.2.4) sp act, the microsomes could account for ca. 10% of the lamellar body protein (7). Mitochondrial

contamination of the lamellar body fraction could not be demonstrated since cytochrome C oxidase, succinate dehydrogenase (EC 1.3.99.1) or monoamine oxidase (EC 1.4.3.4) activities were not present in these lamellar body preparations.

An aliquot of each fraction was frozen in a dry ice-acetone bath (-70 C) and thawed (25 C) 10 separate times. Another aliquot of each subcellular fraction was placed in an ice bath (4 C) while the other aliquots were in the freeze-thaw process and these served as controls. After treatment, all samples were centrifuged at 105,000 x g for 60 min at 3 C and the resulting pellets and supernatant fractions were analyzed for various enzymatic activities.

A lysosomal-enriched fraction was prepared from rat lung and liver tissues by a modification of the Stoffel and Trabert method (12). Male Sprague-Dawley rats, weighing 500-600 g, were injected intravenously via tail vein with 2.0 ml of a solution of Triton WR 1339, Nembutal was injected intraperitoneally, the animals were killed by decapitation and the lungs and livers were excised. The lungs were lavaged as described by Mavis et al. (10). Control rats of same body weight were given Nembutal and killed by decapitation without treatment. The parenchyma was minced, homogenized in ice-cold Tris-sucrose buffer (Tris [10 mM], sucrose [0.25 M] and EDTA [2 mM]), pH 7.4 (5 vol/g wet weight tissue). All subsequent procedures were performed at 0-4 C. The homogenate was centrifuged at 450 x g for 10 min. The resulting supernatant fraction was centrifuged at 12,000 x g for 10 min. The resulting pellet, which contained predominately the mitochondrial and lysosomal fractions, was resuspended in Tris-sucrose-buffer and recentrifuged at 12,000 x g for 10 min. The pellet was resuspended in Tris-sucrose buffer and centrifuged at 450 x g for 10 min and the pellet discarded. The resulting supernatant fraction (2-2.5 ml) was overlayered on a sucrose gradient which consisted of 20% sucrose (1.9 ml) followed by 2.0 ml each of 25, 30, 35, 40, 45 and 50% sucrose on a cushion of 1.5 ml 60% sucrose. All sucrose solutions contained EDTA (2 mM) except the 60% sucrose fraction. Centrifugation was performed at 100,000 x g for 180 min using a SW 27 rotor. After centrifugation, particle fractions found in interfaces between gradients were collected by Pasteur pipette, diluted with Tris-sucrose buffer and centrifuged at 105,000 x g for 60 min. The pellet of each fraction was dissolved in 1.0-3.0 ml Tris-sucrose buffer prior to assay. A total of 8 fractions was obtained. The distribution of the mitochondrial (fractions VI and VII) and microsomal (fractions II and III) fractions in rat lung and liver tissues in this gradient system were determined by the marker enzymes, succinate dehydrogenase and NADPH-dependent cytochrome C reductase, respectively. The sp act of these marker enzymes did not change by Triton WR 1339 administration. N-Acetyl-β-glucosaminidase (EC 2.3.1.30) and β-galactosidase (EC 3.2.1.23) were assayed according to the Peters et al. method (13) employing 4-methylumbelliferyl derivatives as substrates. Latency of N-acetyl-β-glucosaminidase and β-galactosidase activities was demonstrated by assaying the various pellet fractions in the presence and absence of 0.1% Triton X-100. PAPase activity was assayed employing [^{32}P] phosphatidic acid as substrate (7). All assays were performed in duplicate and recoveries of enzymatic activity were greater than 80%, with the exception of the lamellar body β-galactosidase in the control sample (71%). Protein was measured by the Lowry et al. method (14).

RESULTS

The distribution of PAPase activity in the mitochondrial, microsomal and lamellar body fractions prepared from pig lung tissue is presented in Table I. The PAPase sp act in the lamellar bodies (31.5 nmol x min^{-1} x mg^{-1} protein) was significantly greater than that found in the microsomes (16.8) as was previously reported (7). Although the fraction of total tissue PAPase activity that was associated with the lamellar bodies was low (0.3%), it should be recalled that the lamellar bodies are derived only from the type II pneumonocyte, a cell type which comprises 10-15% of the lung cells. On the other hand mitochondrial and microsomal fractions are derived from the total cell population. We have examined the pig lung lamellar body fraction employing transmission electron microscopy. Over 80% of the subcellular organelles in this fraction showed lamellated structure and most of the lamellar bodies contained a perilamellar membrane. No tubular myelin structures were present in this fraction. Based on the dipalmitoyl-phosphatidylcholine content of the lung, we have calculated that ca. 22% of the total lung lamellar bodies were isolated. This recovery of lamellar bodies is similar to that reported by Voelker and Snyder (25%) who determined their isolation recovery of lamellar bodies by the addition of trace quantities of previously radiolabeled lamellar bodies prior to their fractionation procedure (15). Further support for the conclusion that there is enrichment of PAPase activity in the type II pneumonocyte

TABLE I

Release of Enzyme Activities from Porcine Lung Mitochondria, Microsomes and Lamellar Body Fractions after Repeated Freezing and Thawing[a]

	Mitochondria	Microsomes	Lamellar bodies
Glucosaminidase	91.3[b]	8.5	0.3
Control	0.54[c] (14.5)	0.05 (6.6)	0.06 (21.8)
Freeze-thawed	1.33 (36.5)	0.04 (6.1)	0.10 (44.4)
Galactosidase	92.9	6.8	0.3
Control	1.83 (15.4)	0.34 (18.5)	0.22 (27.2)[d]
Freeze-thawed	6.38 (45.5)	0.47 (24.4)	0.39 (45.7)
PAPase	69.0	30.7	0.3
Control	0.45 (0.5)	2.80 (2.9)	0.05 (0.4)
Freeze-thawed	0.90 (1.2)	1.50 (1.5)	0.05 (0.3)

[a]An aliquot of each subcellular fraction was frozen and thawed 10 times while a similar aliquot was maintained at 4 C (control). Each sample was centrifuged at 105,000 x g for 60 min. The resulting pellet and supernatant fractions were assayed for enzyme activities. The values are expressed as substrate hydrolyzed, nmol/min in the supernatant fraction. In parentheses, the percentage of the activity of each enzyme released into the supernatant fraction is presented. Recoveries were greater than 80%.

[b]Percentage of the activity in the total activity found in each subcellular fraction.

[c]This designates nmol product x min^{-1} formed by the action of enzyme found in the supernatant fraction after repeated freeze-thawing and centrifugation at 100,000 x g for 60 min.

[d]Recovery 71%.

was our observation that type II pneumonocytes grown in tissue culture have a significantly higher PAPase sp act than do other lung cells grown under identical conditions (16).

The amount of enzyme activity released from various subcellular fractions into the supernatant fluid after repeated freeze-thawing is presented in Table I. The data are expressed as units of enzyme released and as the percentage of the activity that was released into the supernatant fraction of the freeze-thawed and the control sample. There was greater release of 2 lysosomal marker enzyme activities, viz., N-acetyl-β-glucosaminidase and β-galactosidase from the mitochondrial fraction preparation following the freeze-thaw procedure compared to that released from the mitochondrial fraction maintained at 4 C. These results were expected since this fraction should be enriched with lysosomes. There was no difference in the amount of PAPase activity released from the mitochondrial fractions treated by the freeze-thaw procedure than there was in the mitochondrial fraction kept at 4 C. It can be calculated that freezing and thawing of the mitochondrial fraction resulted in the release of 22% of the total N-acetyl-β-glucosaminadase and 30.1% of the total β-galactosidase activity (freeze-thaw value minus control value). In contrast, only 0.7% of the PAPase activity was released by the freeze-thaw procedure. The microsomal fraction contained a minimal amount of lysosomal marker enzyme activity. In contrast, the microsomal fraction contains a significant amount of PAPase activity.

There was significantly greater release of N-acetyl-β-glucoasminidase and β-galactosidase activities from lamellar bodies treated by freeze-thawing than from lamellar bodies maintained at 4 C; a minimal release of PAPase activity was observed from lamellar bodies after freeze-thawing, a finding that leads us to conclude that the activity of PAPase in the lamellar body preparation cannot be attributed to the contamination of the lamellar body fraction with lysosomes. However, we cannot exclude the possibility that the PAPase activity associated with the lamellar body preparation is of lysosomal membrane origin and that such membranes are pelletized with the lamellar bodies during their purification following the freeze-thaw procedure.

In order to address the question as to whether the lysosomes of lung tissue was enriched with PAPase activity, we prepared a lysosome-enriched fraction of lung tissue from rats which had been pretreated with Triton WR 1339. Triton WR 1339 treatment has been used to obtain a lysosomal-enriched fraction from rat liver which is clearly separated from the mitochondrial and peroxisomal fractions (17).

The 12,000 x g pellet obtained from rat lung and liver tissues containing principally mitochondria and lysosomes was further fractionated by the discontinuous sucrose density gradient centrifugation. Glucosaminidase, β-galactosidase and PAPase activities were determined in each fraction of the sucrose gradient. The results of this experiment are presented in Table II. The distribution of the enzyme

TABLE II

Distribution on Discontinuous Sucrose Gradient of Enzymes in Lung and Liver Lysosome-mitochondrial Fractions Obtained from Control Rats and Rats Treated with Triton

Rat liver	Enzyme activities in fractions I to IV			
	Specific Activity nmol x min^{-1} x mg^{-1} protein		Total recovered activity (%)	
Rat liver				
Galactosidase				
Control (3)[a]	0.76 ± 0.24[b]	$P < 0.001$	0.97 ± 0.31	$P < 0.001$
Triton-treated (5)	13.26 ± 2.88		52.86 ± 10.87	
Glucosaminidase				
Control (3)	1.00 ± 0.37	$P < 0.02$	0.80 ± 0.17	$P < 0.001$
Triton-treated (5)	17.54 ± 7.82		58.66 ± 12.99	
PAPase				
Control (3)	3.93 ± 1.55	$P < 0.005$	2.41 ± 1.00	$P < 0.005$
Triton-treated (5)	19.99 ± 4.39		20.74 ± 5.67	
Rat Lung				
Galactosidase				
Control (4)	2.40 ± 0.51	$P < 0.005$	12.97 ± 1.68	$P < 0.005$
Triton-treated (5)	4.79 ± 0.71		18.98 ± 1.99	
Glucosaminidase				
Control (4)	1.16 ± 0.25	$P < 0.02$	7.23 ± 0.65	$P < 0.001$
Triton-treated (5)	2.46 ± 0.72		13.34 ± 1.93	
PAPase				
Control (4)	37.84 ± 8.11	$P > 0.8$	15.60 ± 2.55	$P > 0.2$
Triton-treated (5)	39.90 ± 11.73		12.54 ± 3.16	

[a]Number of samples analyzed.

[b]Mean ± SD.

activities of the 12,000 x g fraction was compared in liver tissue obtained from control rats and rats treated with Triton WR 1339. It is evident from data presented in Table II that lysosomal marker enzyme activities as well as PAPase were shifted to less dense fractions (fractions 1 to IV) by Triton treatment when liver tissue was employed. Thus, we have confirmed that a significant quantity of PAPase activity in rat liver tissue is associated with the lysosomal fraction (8). A significant amount of the glucosaminidase and β-galactosidase activities in the 12,000 x g fraction of rat lung tissue also migrated to less dense fractions following Triton WR 1339 treatment. This was true for both the specific and the total activities of these fractions. Triton 1339 administration resulted in a less pronounced change in the distribution of known lysosomal enzymes in lung tissue compared to liver tissue.

While a portion of the PAPase activity of rat liver tissue was found to be of lysosomal origin as measured by the shifts in distribution on the gradient after Triton WR 1339 treatment, the distribution of rat lung PAPase activity did not change by such treatment. Therefore, in lung tissue, Triton WR 1339 treatment did not change the density of the particles containing PAPase activity. However, such treatment did effect an alteration in the density of some particles which contained the lysosomal marker enzymes, glucosaminidase and β-galactosidase (Table II). This finding also supports the view that PAPase activity is not principally of lysosomal origin in the lung tissue.

DISCUSSION

It has been demonstrated that various lysosomal enzymes are associated with lamellar bodies (18). In studies designed to define the origin of lamellar bodies of the type II pneumonocyte using morphological and biochemical techniques, results were obtained which support the hypothesis that the morphological precursor of this subcellular organelle is a complex consisting of Golgi apparatus-endoplasmic reticulum-lysosomes [GERL] or multivesicular bodies (19). Although we found a significant amount of lysosomal enzyme activity in the lamellar body fractions, such findings do not allow us to distinguish whether such enzyme activity arose by lysosomal contamination or by contribution of lysosomes to the morphogenesis of lamellar bodies.

We have suggested that the enzyme PAPase is closely associated with isolated lamellar bodies (1) and that the activity of PAPase

cannot be accounted for by contamination of the lamellar body preparation with microsomes (7). From the results of this study, we have demonstrated that PAPase activity is not released by the freezing and thawing of a highly purified lamellar body preparation. Moreover, the distribution of PAPase activity on a discontinuous sucrose density gradient prepared from lung tissue homogenates does not change by the prior treatment of rats with Triton WR 1339, which causes a shift in part in the density of particles containing known lysosomal marker enzymes. These 2 reported observations support the view that the PAPase activity associated with lamellar bodies cannot be accounted for by lysosomal contamination. The highest sp act of lung PAPase on the gradient was found to be fraction IV, which has the density between mitochondria and microsomes. We are presently investigating the biochemical and morphological characteristics of fraction IV as this fraction may relate to the origin of lamellar bodies. Enzymes other than PAPase which are involved in glycerophospholipid metabolism have also been demonstrated in purified lamellar body fractions (20-22). Recently, Baranska and van Golde suggested that the activity of several of these enzymes can be accounted for by microsomal contamination of lamellar body fraction (23).

The physiological role of the PAPase activity associated with the lamellar bodies is unclear. It has been suggested that the function of enzymes of lysosomal origin which are associated with lamellar bodies is to facilitate the exocytosis of the lamellar bodies from the type II pneumonocytes (18). We have suggested that phosphatidate phosphohydrolase may be involved in the regulation of the biosynthesis of the phosphatidylcholine and phosphatidylglycerol found in surfactant (7,24).

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Cyclopropene Fatty Acids in Some Malaysian Edible Seeds and Nuts: I. Durian (*Durio zibethinus*, Murr.)

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ABSTRACT

The aril and seeds of the fruit Durian (*Durio zibethinus*, Murr.) were examined for their protein content and fatty acid composition by gas liquid chromatography (GLC). The values (area percentage) for fatty acids as methyl esters were: aril = 14:0 (0.91%); 16:0 (34.13%); 16:1 (7.10%); 18:0 (1.21%); 18:1 (42.14%); 18:2 (7.85%) and 18:3 plus 20:0 (5.69%), Seeds = 14:0 (0.12%); 16:0 (12.20%); 16:1 (1.15%); 18:0 (1.42%); 18:1 (8.42%); 18:2 (6.50%); dihydrosterculic acid (2.52%); 18:3 plus 20:0 (11.30%); malvalic acid (15.72%); sterculic acid (38.53%) and 22:0 (1.21%). The germ oil contained the highest amount of sterculic acid. The cooking temperatures employed reduced the malvalic and sterculic acid contents in seeds only by ca. 22% and 19%, respectively.

INTRODUCTION

Durian, *Durio zibethinus*, Murr. (*Bombacaceae*) is cultivated in its natural habitat largely for its fruit throughout Southeast Asia. The fruits are highly prized and are much appreciated for their odor and delicate flavor by the people of this region. The edible portion is the aril in the fruit; the seeds may also be consumed. The aril is normally eaten fresh, sometimes fermented with salt ("tempoyak") or boiled with sugar ("lempok"). The flavor of the fruit changes with age. As soon as the fruit falls, very rapid chemical changes take place in the aril. These changes continue to a stage when the aril can no longer please the ordinary palate. It is said that the fruit cannot be kept for more than 5-6 days without losing its characteristic flavor and texture. Experiments at prolonging the shelf life by cold storage have had only partial success (1). However, the aril along with seeds could be blanched, frozen and stored below -10 C for as long as 6 m without any easily detectable organoleptic changes (S.K. Berry, personal communication, 1976).

The seeds are embedded in the arils. They are large, 5-7 cm long by ca. 2-4 cm wide, hard but not stony and are covered with a thin, light-brown skin. A fresh seed may weigh as much as 40 g. The seeds are boiled or baked prior to consumption.

Stanton (2) gave a full description of the fruit and reviewed its proximate analyses. Recently, volatile flavoring constituents of the aril were reported (3). The seeds contain very little oil. The fresh aril, however, contains as much as 5% of an oil with unknown fatty acid composition. Since the fruit deteriorates rapidly and loses its edible value, it would be of practical and scientific interest to investigate the fatty acid composition of the aril. Additionally, the seeds may contain cyclopropene fatty acids (CPFA) that are commonly found in plants of the order Malvales to which durian belongs (4). The adverse biological effects of these fatty acids in farm and experimental animals are well documented (5-9). More recently, CPFA were found to cause cancer in rainbow trout (10) and aortic atherosclerosis in rabbit (11). These observations are of great concern because humans unknowingly consume seeds and nuts which may contain these fatty acids.

The aim of this study was to explore the fatty acid composition of both aril and seeds of the fruit durian and to investigate the influence of cooking temperature on the fate of cyclopropene fatty acids in seeds.

EXPERIMENTAL PROCEDURES

Durians were purchased from the local market and prepared for analysis immediately. Fatty acid methyl ester reference standards were obtained through Sigma Chemical Company (St. Louis, MO). Sodium methoxide reagent (0.5 N) was purchased from Supelco, Inc. (Bellefonte, PA). All other reagents used were of analytical grade.

Extraction of Oil and Analyses

The freeze-dried aril pulp and oven-dried seeds were pulvarized to a fine powder and extracted separately with petroleum ether (bp 40-60 C) in Soxhlet apparatus for 16 hr. The oil was recovered by evaporating the ether on a rotary evaporator under reduced pressure.

A sample of durian seeds from the same lot was boiled in water until soft. The boiled seeds were then dried and extracted for oil in the same manner.

Moisture content (air oven method) and protein content (Kjeldahl method) of both aril

and seeds were determined as described by AOAC (12). The Halphen color test was performed according to the Coleman and Firestone method (13).

Preparation of Methyl Esters and Silver Nitrate Derivatives

Methyl esters of the oil fatty acids were prepared by transmethylation using sodium methoxide (0.5 N) in methanol as described by Timms (14). The contents of the reaction vessel were centrifuged for clarification. For seed oils, the petroleum ether layer containing the methyl esters was removed and treated with methanol saturated with silver nitrate according to the Schneider et al. method (15) to obtain stable CPFA ester derivatives. The normal fatty acid esters and the CPFA ester derivatives were recovered from the reaction mixture in the usual manner.

Gas Liquid Chromatography

The mixture of fatty acid methyl esters derived from their respective oils was analyzed on a Pye Series 204 gas chromatograph equipped with hydrogen flame ionization detectors. The analysis was performed on 2 glass columns of the same length (1.5 m x 4 mm, id) but containing 2 different phases of opposite polarity. Column A, which was packed with 10% w/w polyethylene glycol succinate adsorbed on 100-120 mesh Diatomite CAW, was operated at 180 C with nitrogen as carrier gas (OFN) at a flow rate of 30 ml/min. Column B, which contained 10% w/w APL supported on 100-120 mesh Diatomite CAW DMCS, was operated at 220 C with nitrogen as carrier gas at a flow rate of 50 ml/min. The injection port and detector temperatures were maintained at 200 C.

Gas chromatograph peaks were identified by comparison with pure methyl esters through retention time relative to methyl heptadecanoate on 2 columns. The oil of *Sterculia foetida* seeds was used as reference standard to identify cyclopropene fatty acids. The fatty acid methyl ester derivatives of this oil were mixed with sample fatty acid ester derivatives and chromatographed together (cochromatography) on column A.

The area percentage of each peak was obtained on Hewlett-Packard 3380A Integrator linked directly to the gas chromatograph. The analysis was performed in duplicate.

Infrared (IR) spectra of fatty acid methyl esters were determined on a Beckman 4240 IR Spectrophotometer. A Varian Techtron Model 635 spectrophotometer was used to record ultraviolet (UV) spectra.

RESULTS AND DISCUSSION

Aril Oil

The dried aril yielded ca. 15% of a yellow-colored oil. The oil did not respond to Halphen test indicating the absence of CPFA. The fatty acid composition of the aril is given in Table I. The oil from 2 durian strains, namely with cream- or yellow-colored aril, exhibited variation in fatty acid composition as evident from the data in Table I. The oil, however, was primarily composed of palmitic and oleic acids which predominate in most fruit-coat fats (16). The unsaturated fatty acids constituted ca. 62-70% of the total fatty acids. The linolenic acid content, especially in the yellow-colored aril, was relatively higher compared to most fruit-coat fats.

The aril is normally consumed immediately after opening the fruit—otherwise it loses its flavor and firm, creamy texture and becomes rancid and watery. These changes in flavor and texture may be attributed to oxidative and hydrolytic alterations in fatty acid composition of the fat. The chemical composition of the lipid invariably influences the textural properties of fat-containing foods (17). The products of oxidation, and hydrolysis of the unsaturated fatty acids in particular, make the fruit taste rancid thus upsetting the delicate flavor balance. The fresh aril, however, provides a sufficient amount of essential fatty acids to the consumer.

Seed Oil

Both fresh and boiled seeds were examined for their oil content (1.8%). The fatty acid composition of the whole seeds, germ and cotyledons is presented in Table I. The whole seed oil contained ca. 82% unsaturated fatty acids. The palmitic acid content of this oil was relatively lower compared to other seed oils of the family Bombacaceae (16). Of the unsaturated fatty acids, linoleic and linolenic together constituted ca. 22%. The cooking temperatures employed did not significantly affect the concentration of these fatty acids in seeds.

Occurrence of Cyclopropene Fatty Acids

The oil samples from the whole seeds, germ and cotyledons gave a positive Halphen test indicating the presence of CPFA. This was further supported by IR studies. The methyl esters of oil fatty acids had the characteristic IR band for cyclopropene moiety at 1008 cm^{-1}. The spectrum, however, did not exhibit the presence of a hydroxyl or terminal acetylenic group. The UV spectra showed no conjugation in the oils.

TABLE I

Analytical Data on Durian Aril, Seed and Their Oil

Property	Value					
			Seeds			
	Aril		Whole			
	Yellow	Creamy	Fresh	Cooked	Germ	Cotyledons
Composition (%)						
Moisture	66.00	ND[a]	77.00	61.60	77.00	77.00
Oil[b]	5.00	ND	0.50	0.50	1.54	0.20
Protein[b] (N x 6.25)	2.50	ND	1.57	ND	4.23	ND
Oil fatty acid composition (Area %)						
14:0	0.91	0.34	0.12	0.17	0.12	0.25
16:0	34.13	28.94	12.20	22.81	4.52	14.90
16:1	7.10	5.16	1.15	1.07	0.26	1.00
18:0	1.21	1.23	1.42	1.66	0.41	2.00
18:1	42.14	58.98	8.42	7.67	3.92	9.84
18:2	7.85	3.16	6.50	6.26	4.07	6.50
Dihydrosterculic[c]	---	---	2.52	3.09	1.66	4.05
18:3	5.69	2.21	11.30	13.61	5.62	16.64
20:0	Trace[d]	Trace				
Malvalic	---	---	15.72	12.28	1.76	20.65
Sterculic	---	---	38.53	31.34	77.68	20.89
22:0	---	---	1.21	Trace	---	2.82

[a]ND = Not Determined.

[b]Wet basis.

[c]Tentative identification.

[d]Trace = < 0.1%.

The presence of malvalic and sterculic acids was confirmed by comparison with silver nitrate derivatives of fatty acid methyl esters of *Sterculia foetida* seed oil through retention time and cochromatography on column A. Dihydrosterculic acid was tentatively identified in all these oils. These oils were found to contain a rather large amount of CPFA in which sterculic acid predominates; the highest amount is in germ oil. The ratio of sterculic acid-to-malvalic acid in the oil of whole seeds, germ and cotyledons was found to be ca. 2:1, 44:1 and 1:1, respectively. The germ was examined separately for its CPFA content because the removal of the germ from seeds lessens the effect of skin irritation, as experienced by some consumers. Furthermore, the seeds have been reported to be indigestible and contain a poisonous substance which causes shortness of breath (2). These deleterious properties could probably be attributed to the excess of sterculic acid, which has been implicated in exerting stronger biological effects in animals (5, 18).

Effect of Heat on CPFA

Cyclopropene fatty acids are quite labile to heat and tend to undergo polymerization. It was observed that, in the Halphen test on the cooked seed oil, the color development was rather slow. This could have resulted from partial polymerization (which is reversible under alkaline conditions) of CPFA during cooking (4). Quantitative studies showed that the malvalic and sterculic acid contents of whole seed decreased upon cooking only by ca. 22% and 19%, respectively. It is ambiguous whether CPFA still exhibit their biological activity after such heat treatments. The isolated seed oils containing CPFA, however, do not respond to the Halphen color test and at the same time lose their biological activity when subjected to high temperatures (19,20).

CONCLUSION

The foregoing results showed that the aril is a good source of essential fatty acids, besides having aesthetic value. The seeds, on the other hand, contain a high amount of undesirable cyclopropene fatty acids which are only partially destroyed during cooking. It would therefore seem extremely unwise to consume these seeds.

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Abnormal Suppression of 3-Hydroxy-3-Methylglutaryl-CoA Reductase Activity in Cultured Human Fibroblasts by Hypertriglyceridemic Very Low Density Lipoprotein Subclasses[1]

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ABSTRACT

Our previous studies showed that hypertriglyceridemic very low density lipoproteins (HTG VLDL) are functionally abnormal. HTG VLDL, but not normal VLDL, suppress HMG-CoA reductase in cultured normal human fibroblasts. To determine if the suppression by HTG VLDL resulted from a subpopulation of smaller suppressive particles, more homogeneous subclasses of VLDL-$VLDL_1$ (S_f 100-400), $VLDL_2$ (S_f 60-100), and $VLDL_3$ (S_f 20-60) were obtained from the $d < 1.006$ ($g \cdot ml^{-1}$) fraction of normal and hypertriglyceridemic plasma by flotation through a discontinuous salt gradient and tested for suppression in normal human fibroblasts. $VLDL_1$ and $VLDL_2$ from each of the 12 normolipemic subjects tested failed to suppress HMG-CoA reductase activity in normal fibroblasts. Eleven out of 12 preparations of normal $VLDL_3$ suppressed HMG-CoA reductase, but only one-third as effectively as LDL. By contrast, the $VLDL_1$, $VLDL_2$ and $VLDL_3$ from 15 out of 17 hypertriglyceridemic patients (hyperlipoproteinemia Types IIb, III, IV and V) were highly effective in suppression, with half-maximal suppression at 0.1-2.0 μg VLDL protein/ml. The VLDL abnormality is apparently associated with hypertriglyceridemia and not hypercholesterolemia, since VLDL from a homozygous familial hypercholesterolemia patient with a Type IIa pattern did not suppress whereas each of the VLDL subclasses from a Type IIb patient suppressed. Suppression by HTG VLDL in normal cells is apparently a consequence of interaction of the protein portion of the VLDL with the specific LDL cell surface receptor since HTG $VLDL_1$ treated with 0.1 M 1,2-cyclohexanedione to block arginyl residues failed to suppress the enzyme. Moreover, hypertriglyceridemic S_f 60-400 VLDL failed to suppress HMG-CoA reductase activity in LDL receptor-negative fibroblasts. There were no consistent major compositional differences between comparable normal and hypertriglyceridemic VLDL subclasses which could account for differences in suppression. All VLDL subclasses from Type III subjects were enriched in cholesteryl esters and depleted in triglyceride, relative to the corresponding normal VLDL subclasses. However, Type IV and Type V VLDL subclasses were normal in this respect. We conclude from these studies that small particle diameter is not required for suppression, since HTG $VLDL_1$ and $VLDL_2$ which contained few, if any, small particles were effective in suppression.

INTRODUCTION

Triglycerides in the plasma are carried primarily in chylomicrons, particles formed in the intestine with triglycerides of exogenous origin, and in very low density lipoproteins (VLDL), particles formed mainly in the liver with endogenously synthesized triglycerides. Chylomicrons are not found in the plasma of normal, fasting man; VLDL are the only triglyceride-rich lipoprotein present and these are at low levels. Large VLDL are converted in vivo to smaller VLDL "remnants" and finally to low density lipoproteins (LDL) primarily as a result of hydrolysis of the triglycerides by lipoprotein lipase. A significant portion of the plasma LDL are probably catabolized by nonhepatic cells by a pathway first identified in cultured human fibroblasts by Goldstein and Brown (1). In cultured normal cells, LDL bind to a specific cell surface receptor, are internalized by endocytosis and degraded in the lysosomes. After specific LDL degradation, cellular cholesterol esterification is enhanced and the synthesis of the receptor and of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase are suppressed, thus limiting both cellular uptake of LDL and biosynthesis of cholesterol. Mahley and coworkers have shown that certain high density lipoproteins which contain apoprotein E (apo E) also bind with high affinity to the LDL receptor and suppress HMG-CoA reductase activity (2). Modification with cyclohexanedione of the arginyl residues of apoprotein B (apoB) in LDL and apoE in the suppressive HDL abolishes the ability of the particles to interact with cells (3). Our previous studies showed that VLDL isolated from fasting

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normolipemic plasma by rate zonal ultracentrifugation do not suppress HMG-CoA reductase activity in cultured normal human fibroblasts even though they contained both apoB and apoE (4). In contrast, VLDL isolated by the zonal method from the plasma of hypertriglyceridemic patients with hyperlipoproteinemia Types III, IV and V suppress HMG-CoA reductase in normal, but not in receptor-negative fibroblasts (4). The molecular basis for this difference in unknown. The normal VLDL apparently contain all of the structural elements necessary for receptor-mediated suppression, but in a masked form, since the nonsuppressive normal VLDL can be converted with purified lipoprotein lipase in vitro to LDL-like particles (VLDL remnants) which are as suppressive as native LDL in normal, but not in receptor negative, fibroblasts (5).

An obvious and major difference between LDL and VLDL is size. A potential explanation for the differences in suppression by normal vs hypertriglyceridemic VLDL is that the suppressive, hypertriglyceridemic VLDL contain a subpopulation of smaller particles which are potent suppressors. Since the total VLDL fraction contains particles which are heterogeneous with respect to size, density and composition, we isolated more homogeneous subclasses of VLDL, $VLDL_1$ (S_f 100-400), $VLDL_2$ (S_f 60-100), and $VLDL_3$ (S_f 20-60) (6) from 12 normolipemic and 17 hypertriglyceridemic subjects. We tested these fractions for their effects on HMG-CoA reductase activity in normal human fibroblasts.

Our studies show that even $VLDL_1$, the largest VLDL particles, from 15 of the 17 hypertriglyceridemic subjects effectively suppress HMG-CoA reductase in cultured normal fibroblasts. In contrast, of the normal VLDL subclasses, only the smallest–S_f 20-60 VLDL–exhibited any ability to suppress HMG-CoA reductase and this suppression was moderate. Smaller lipoprotein size may be an important factor in suppression, but it is neither the sole determinant nor an essential one.

METHODS

Materials

DL-3-Hydroxy-3-methyl[3-^{14}C]-glutaryl-CoA (20-50 Ci/mol) was obtained from New England Nuclear (Boston, MA). NADP, D-glucose-6-phosphate (monosodium salt) and glucose-6-phosphate dehydrogenase (Type XV, sulfate-free) were purchased from Sigma Chemical Company (St. Louis, MO). 1,2-Cyclohexanedione was obtained from Aldrich Chemical Company (Milwaukee, WI). Reagent grade hydroxylamine, sodium borate and mannitol were purchased from Fisher Scientific Company (Pittsburgh, PA). Thin layer chromatographic materials and tissue culture supplies were obtained as previously reported (4).

Cells

Monolayer cultures of normal human fibroblasts were established from a preputial specimen obtained from a healthy newborn. Cells were maintained in 100 mm dishes in a humidified incubator (5% CO_2) at 37 C and used between the third and ninth passage. A detailed description of the growth conditions and the subculture method has been presented (4). For experimental purposes, ca. 5 x 10^4 cells/dish were seeded into dishes (60 x 15 mm) containing complete medium (3 ml). After 3 days of growth, when the cells were ca. 75% confluent, the medium was removed; the cells were washed with 3 ml saline and were placed on 2 ml medium containing 5% human lipoprotein-deficient serum (LPDS) for 24 hr, to allow HMG-CoA reductase activity to increase to unsuppressed levels. Indicated quantities of lipoproteins (in 0.2 ml) were then added to duplicate dishes and incubated for 16 hr. The medium was then removed, the cell monolayers washed twice at room temperature with 2 ml saline, and the cells scraped with a rubber policeman into 2.0 ml 0.15 M NaCl containing 50 mM Tris-HCl, pH 7.4. The cells were sedimented by centrifugation, the supernatant was discarded and the cell pellets stored at -80 C.

HMG-CoA Reductase Assay

HMG-CoA reductase activities were determined by a modification of the Brown et al. method (7). Cell pellets were thawed rapidly and incubated for 10 min at 37 C with 0.1 ml 50 mM K_2HPO_4, pH 7.4, containing 5 mM dithiothreitol, 1 mM EDTA, 0.15 M KCl and 0.25% Kyro EOB (Proctor and Gamble, Co., Cincinnati, OH). After centrifugation for 1 min in a Beckman Microfuge at room temperature, the clear supernatants were assayed in duplicate for HMG-CoA reductase activity. Cell extracts (20 μl) were incubated at 37 C in a final vol of 35 μl with 3 mM NADP; 22 mM glucose-6-phosphate; 14 mM Tris-HCl, pH 7.5; 5 mM dithiothreitol, 0.15 unit of glucose-6-phosphate dehydrogenase; and 43 μM (3-^{14}C]-HMG-CoA. After 2 hr, 10 μl of 2.5 N HCl containing 60 mM mevalonolactone as carrier was added. After 20 min at 37 C, an aliquot (15 μl) of the acidified reaction mixture was streaked on a silica thin layer chromatogram (8). The chromatograms were air-dried and developed in acetone/benzene (1:1, v/v) until the solvent

front had moved 5.5 cm above the origin (the top of the chromatogram). Areas of the chromatogram containing mevalonolactone (R_f 0.63) and residual HMG-CoA (origin) were counted in a liquid scintillation counter. Each HMG-CoA reductase activity data point is the average of duplicate determinations of duplicate dishes, which differed by less than 15%. Protein content of extracts was determined by the Lowry et al. method (9) using bovine serum albumin as a standard.

Lipoproteins and Lipoprotein Deficient Serum (LPDS)

Normal lipoprotein fractions and LPDS (d > 1.21) were isolated from the plasma of fasting (12-14 hr) adult normolipemic males (age 21-26, plasma triglyceride levels ≤ 110 mg/dl, and cholesterol ≤ 200 mg/dl, no known medical disorders, receiving no drugs). Hyperlipoproteinemic VLDL were obtained from the plasmas of patients with hyperlipoproteinemia Types IIA, IIB, III, IV and Type V. The diagnoses were based on commonly used criteria (10). One Type III patient has been described in detail elsewhere (4). The plasma cholesterol and triglyceride levels expressed as mg/dl of the hypertriglyceridemic subjects were as follows: Type IIb, 271 and 244; Type III, 374 and 480, 234 and 187, 316 and 344; Type IV, 261 and 477, 255 and 368, 194 and 291, 190 and 363, 187 and 216, and 165 and 374; Type V, 670 and 4330, 312 and 1589, 371 and 2824, 282 and 1520, 285 and 732, 282 and 1260, and 359 and 1776. The types IIB, III and IV subjects were on no special lipid-lowering diets or drug therapies for at least 4 wk prior to lipoprotein isolation; plasma lipid levels were similar 1 wk prior to plasma donation. In the Type IIb, III and IV subjects, clinical, biochemical and hematologic studies were used to exclude secondary causes of hyperlipoproteinemia. Seven of the subjects (Types III and IV) have been described in detail elsewhere (11). The Type V subjects probably could not be considered to be in a steady state with respect to plasma triglyceride levels at the time plasma was obtained for lipoprotein isolation; recent previous lipid values are unknown. Six of the 7 Type V subjects were receiving insulin; one of the diabetic Type V subjects was also taking Atromid-S. One subject was a primary Type V (plasma cholesterol 282 mg/dl, triglycerides 1,260 mg/dl), suffered from coronary heart disease, and was taking rerserpine, digoxin, potassium supplement and nicotinic acid. However, the VLDL subclasses from each of the type V subjects were similarly suppressive, giving half-maximal suppression at 1-2 μg protein/ml. None of the subjects noted any marked weight change in the month prior to plasma donation. Blood from most normal subjects was collected in 0.1% EDTA after a 12-hr fast; red cells were removed by low speed centrifugation. Plasma from several normolipemic and all hyperlipoproteinemic subjects was obtained by plasmapheresis after a 12-14-hr fast. Lipoprotein fractions were isolated from plasma according to standard techniques by sequential flotation in a 60 Ti rotor at 45,000 rpm and 14 C for indicated times in a Beckman preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) with KBr for density adjustment (12) after chylomicrons were removed (0.5 hr at 35,000 rpm). Total VLDL were than isolated in a second centrifugation (18 hr) without adjusting the density of plasma (d < 1.006); LDL were isolated (18-hr spin) at d 1.006-1.063. VLDL (d < 1.006) from a homozygous familial hypercholesterolemia patient with a Type IIa lipoprotein pattern were purified by rate zonal ultracentrifugation in a Beckman Ti-14 zonal rotor; a linear gradient in the density range of 1.000-1.300 (NaBr) was used for ultracentrifugation at 15 C and 42,000 rpm for 140 min (13). $VLDL_1$ (S_f 100-400), $VLDL_2$ (S_f 60-100) and $VLDL_3$ (S_f 20-60) subclasses were isolated from the total VLDL fraction (d < 1.006) by flotation in a density gradient (d 1.006-1.05) in a SW-41 rotor (23 C, 35,000 rpm for 144 min [S_f 100-400], 108 min [S_f 60-100] and 18 hr, 38 min [S_f 20-60]) as described by Lindgren and associates (6). Before subfractionation, most normolipemic VLDL preparations were concentrated by ultrafiltration or in dialysis tubing against dry Sephadex G-75. Separation of VLDL on the basis of size was validated by electron microscopy after negative staining with 2% potassium phosphotungstate (14). The mean diameters of S_f 100-400 VLDL, S_f 60-100 VLDL, S_f 20-60 VLDL and LDL were 48.9 ± 2.2, 38.0 ± 3.4, 32.7 ± 3.9 nm and 24.6 ± 35 nm (mean ± SD), respectively.

Lipoproteins and LPDS were dialyzed against 3 changes of 50 vol of 0.15 M NaCl containing 50 mM Tris-HCl, pH 7.4, and 0.3 mM EDTA at 4 C for 36-48 hr. Lipoproteins and thrombin-treated LPDS (15) were sterilized by filtration through a 0.2-μm filter unit (Millipore); LPDS was stored at -20 C and lipoproteins at 4 C.

Total protein content of the lipoproteins was determined by modification of the Lowry method; sodium dodecyl sulfate, at a final concentration of 0.1%, was included to prevent interference by opalescence and light scattering (16). ApoB was determined as the difference

between the total protein and the protein soluble in 4.2 tetramethylurea (TMU) (17). Soluble apoprotein composition was determined after delipidation with 4.2 M TMU. The apoproteins were separated by electrophoresis on 7.5% polyacrylamide gels containing 8 M urea, stained with Amido-Schwartz and quantitated by scanning densitometry (17). Cholesterol, free and esterified, was quantified enzymatically (18). Phospholipid phosphorus was assayed by the Bartlett method (19).

1,2-Cyclohexanedione was used to modify the arginyl residues of the lipoproteins and hydroxylamine to remove the adduct exactly as described by Mahley and coworkers (3).

RESULTS AND DISCUSSION

VLDL are heterogeneous with respect to size, density and composition (20). We therefore isolated more homogeneous subclasses of VLDL from normal and hypertriglyceridemic subjects to determine whether or not the abnormal HMG-CoA reductase suppression observed with the total hypertriglyceridemic VLDL were confined to a smaller, "remnant" VLDL subclass or present throughout the VLDL spectrum.

Diameters of the VLDL subclasses were estimated by electron microscopy of negatively-stained particles. Electron micrographs revealed few, if any, small particles in the $VLDL_1$ and $VLDL_2$ subclasses from both hypertriglyceridemic patients and normolipemic subjects. The VLDL subfractions gave monoexponential decay functions by laser light scattering spectroscopy, indicating that the particles in each isolated subclass were homogeneous with respect to size (11).

$VLDL_1$ and $VLDL_2$ from each of the 12 normolipemic subjects tested failed to suppress HMG-CoA reductase activity in normal fibroblasts. Eleven out of 12 $VLDL_3$ preparations from normal plasma suppressed HMG-CoA reductase, but not as effectively as LDL (Fig. 1); one normal $VLDL_3$ sample had no effect on reductase activity. In some experiments, $VLDL_1$ or the combined $VLDL_1$ + $VLDL_2$ from normal subjects stimulated HMG-CoA reductase activity.

In contrast to normal VLDL subclasses, $VLDL_1$, $VLDL_2$ and $VLDL_3$ from 15 patients with hypertriglyceridemia were highly effective in suppression, with half-maximal suppression ranging from 0.1 to 2.0 μg VLDL protein/ml with different VLDL preparations (Fig. 2). The suppressive VLDL subclasses were from patients with hyperlipoproteinemia Types IIb, 1; III, 3; IV, 4; and V, 7. Of the 17 hypertriglyceridemic VLDL donors, only 2 had VLDL subclasses with normal effects on reductase. These 2 donors were primary Type IV patients with plasma triglyceride levels of 368 and 477 mg/dl. The $VLDL_1$ and $VLDL_2$ from 2 other Type IV patients with plasma triglycerides 291 and 363 mg/dl were moderately suppressive whereas their $VLDL_3$ were as potent as LDL in suppression. Each of the 3 VLDL subclasses obtained from 2 additional Type IV patients (plasma triglycerides 216 and 374 mg/dl) were as suppressive as LDL (Fig. 3). There were no

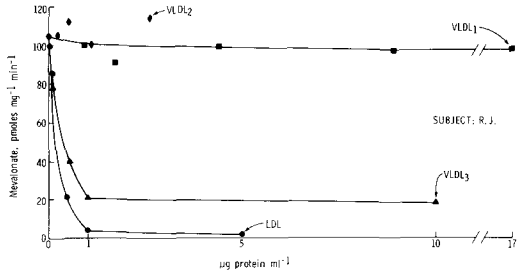

FIG. 1. Effects of normal VLDL subclasses and LDL on HMG-CoA reductase activity in normal human fibroblasts. Cells were grown to ca. 75% confluency in complete medium containing 10% fetal calf serum, washed and placed on 2 ml of medium containing 5% LPDS for 24 hr, as described in Methods. Indicated amounts of lipoproteins, in 0.2 ml, were added to duplicate dishes for 16 hr before the cells were washed and harvested. Each data point is an average of duplicate determinations of HMG-CoA reductase activity in cells from duplicate dishes. Normal LDL (●—●); normal $VLDL_1$ (■—■); $VLDL_2$ (◆—◆); and $VLDL_3$ (▲—▲), purified by flotation through a density gradient d 1.006-1.05, as described in Methods.

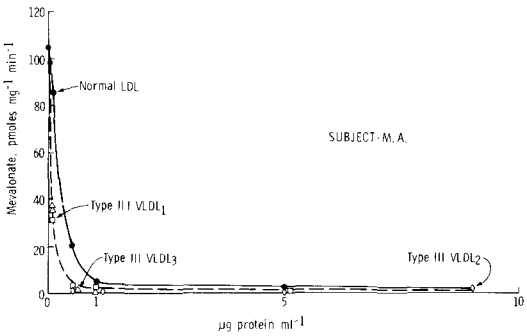

FIG. 2. Effects of hypertriglyceridemic VLDL subclasses on HMG-CoA reductase activity in subconfluent cultures. Growth of the cells and the experimental design were the same as described in the legend to Fig. 1. Each data point is the average of duplicate determinations of duplicate dishes. The hypertriglyceridemic VLDL subclasses were from a Type III subject. Normal LDL (●—●); $VLDL_1$ (□—□); $VLDL_2$ (◇—◇); $VLDL_3$ (△—△).

apparent reasons for these differences among Type IV subjects; however, heterogeneity in cellular effects of the VLDL is consistent with variable etiologies of the Type IV profile.

The observation that hypertriglyceridemic $VLDL_1$ suppressed indicates that small lipoprotein size is not required for suppression. Small particle diameter may contribute to a lipoprotein's ability to suppress, however, since of the normal VLDL subclasses, only the smallest "remnant" $VLDL_3$ suppressed.

Two lines of evidence indicate that the suppression of HMG-CoA reductase activity in normal fibroblasts by hypertriglyceridemic VLDL is mediated by the LDL cell surface receptor. First, hypertriglyceridemic VLDL, like LDL, do not suppress the enzyme in mutant receptor negative cells. Second, as Mahley has shown for LDL and suppressive HDL_c (3), modification of the arginyl residues of hypertriglyceridemic VLDL with 1,2-cyclohexanedione abolishes the ability of the particles to suppress. After removal of the adduct with hydroxylamine, the hypertriglyceridemic VLDL once again suppress HMG-CoA reductase activity (Fig. 4).

The chemical compositions of VLDL subclasses are given in Table I. Each of the VLDL subclasses from the Type III subjects contained more cholesteryl ester and less triglyceride than the comparable subclasses obtained from normolipemic subjects. This abnormality is most striking in the largest subclass, where Type III $VLDL_1$ averaged 28% cholesteryl ester and 50% triglyceride and normal $VLDL_1$ was 6% cholesteryl ester and 64% triglyceride. The VLDL subclasses isolated from Type IV and Type V subjects, however, were similar to normal VLDL subclasses in triglyceride and cholesteryl ester content. The mean values reveal a trend toward slightly higher phospholipid contents and phospholipid:cholesterol ratios and somewhat lower cholesteryl ester contents in the normal VLDL subclasses than the corresponding hypertriglyceridemic VLDL subclasses. However, there were considerable variations in these values among samples, as evidenced by the relatively large standard deviations. Several suppressive Type IV and Type V VLDL preparations had higher phospholipid contents and phospholipid:cholesterol ratios and lower cholesteryl ester contents than did some normal, nonsuppressive VLDL samples. Hence, suppression does not correlate merely with decreased total phospholipid content and/or increased cholesterol or cholesteryl ester content. ApoB, apoE and apoC were detected in each VLDL subclass analyzed, whether normal or hypertriglyceridemic. When

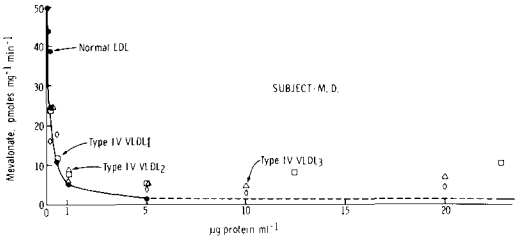

FIG. 3. Effects of Type IV hypertriglyceridemic VLDL subclasses on HMG-CoA reductase activity in normal fibroblasts. Growth of the cells and the experimental design were the same as described in the legend to Fig. 1. Each data point is the average of duplicate determinations of HMG-CoA reductase activity in cells from duplicate dishes. The VLDL were isolated from the plasma of a subject with primary Type IV hyperlipoproteinemia. Normal LDL (●—●); $VLDL_1$ (□—□); $VLDL_2$ (◇—◇); and $VLDL_3$ (△—△) were isolated as described in Methods.

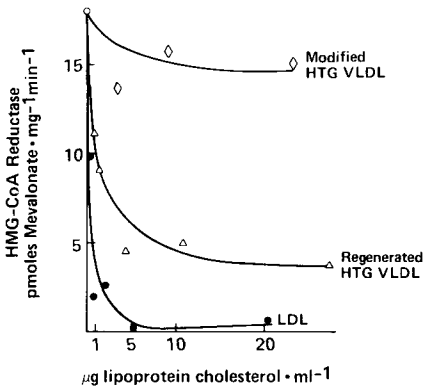

FIG. 4. Role of arginine residues of hypertriglyceridemic VLDL in suppression of HMG-CoA reductase activity. Growth of the cells and the experimental design were the same as described in the legend to Fig. 1 and in Methods. Each data point is the average of duplicate determinations of enzyme activity in cells from duplicate dishes, which differed by less than 10%. The $VLDL_1$ were from a Type V subject. Normal LDL (●—●); HTG $VLDL_1$ treated with 1,2-cyclohexamedione (◇—◇); and regenerated HTG $VLDL_1$, from which the cyclohexane adduct had been removed with hydroxylamine (△—△), as described in Methods.

expressed in terms of percentage of particle weight, as listed in parentheses in Table II, rather than percentage of total protein, the apoprotein contents of the different types of VLDL were similar, except that each of the Type III $VLDL_1$ and one Type V $VLDL_1$ preparation contained less apoC than did normal or Type IV $VLDL_1$. The reason most of the protein in the Type V VLDL subclasses listed is apoB is unknown but apparently is unrelated to the moderate suppression by these

TABLE I

VLDL Fraction	Phenotype	Percentage Weight of Component[1]				
		Protein	Phospholipid	Cholesterol	Cholesteryl ester	Triglyceride
$VLDL_1$						
Type III	(N = 3)	2.8 ± 0.7	12.2 ± 3.3	7.6 ± 1.2	27.6 ± 1.6	49.7 ± 3.3
Type IV	(N = 4)	5.0 ± 1.0	15.5 ± 1.4	5.8 ± 1.1	8.4 ± 1.9	65.4 ± 3.4
Type V	(N = 2)	5.1 ± 2.7	14.7 ± 1.2	5.3 ± 0.9	10.2 ± 0.9	64.7 ± 2.0
Normal	(N = 5)	5.9 ± 0.7	18.5 ± 1.9	5.9 ± 1.4	6.1 ± 1.6	63.6 ± 2.6
$VLDL_2$						
Type III		6.1 ± 0.4	18.6 ± 0.6	7.8 ± 1.8	23.5 ± 4.5	44.0 ± 4.7
Type IV		7.2 ± 1.2	18.8 ± 2.7	6.7 ± 0.3	11.9 ± 4.9	55.3 ± 5.1
Type V		9.4 ± 1.1	19.2 ± 5.0	6.6 ± 1.4	11.5 ± 1.3	53.3 ± 3.3
Normal		8.7 ± 1.4	21.8 ± 3.3	5.8 ± 1.0	8.2 ± 2.9	55.6 ± 3.9
$VLDL_3$						
Type III		9.7 ± 1.0	22.2 ± 2.6	7.9 ± 1.8	32.7 ± 1.4	27.4 ± 1.4
Type IV		10.8 ± 1.4	20.6 ± 2.3	7.0 ± 0.8	19.6 ± 5.5	42.0 ± 6.6
Type V		13.5 ± 1.9	20.4 ± 2.9	5.8 ± 1.0	16.4 ± 1.8	43.9 ± 3.8
Normal		11.4 ± 4.6	23.8 ± 3.3	7.5 ± 1.8	15.9 ± 4.5	41.3 ± 2.6

[1]The total weight of each particle was taken as the sum of the amounts of free cholesterol, esterified cholesterol, triglyceride, phospholipid and total Lowry protein. The values given are the means ± SD.

VLDL; similar apoprotein compositions were obtained with nonsuppressive VLDL subclasses from one normolipemic subject. Because chromogenicities of the apoproteins differ (17), the apoprotein concentrations given in Table II must be considered approximate and not absolute. Therefore, as with the zonally-isolated VLDL, there were no major consistent compositional differences between the comparable normal and abnormal VLDL subclasses of different hyperlipoproteinemia types which could account for differences in suppression. Differences in apoprotein aggregation in the surface film or penetration into the lipoprotein core which may be responsible for differences in cellular effects would not necessarily be reflected in quantitative differences in apoprotein content. Likewise, the presence in the suppressive hypertriglyceridemic VLDL subclasses of a highly suppressive subpopulation would not necessarily be reflected in a dramatic change in composition.

Several observations strengthen our earlier suggestion (4) that the ability to suppress may result from the presence of chylomicron remnants in the VLDL of hypertriglyceridemic subjects. Such remnants are known to be present in VLDL in Type III and Type IV hyperlipoproteinemia (21). In addition, the VLDL abnormality appears to be associated with hypertriglyceridemia and not hypercholesterolemia. The total VLDL from a homozygous familial hypercholesterolemia patient with a Type IIa pattern (elevated plasma cholesterol, normal triglyceride levels) failed to suppress even at 20 μg protein/ml medium. By contrast, each of the VLDL subclasses from a Type IIb (elevation of both cholesterol and triglyceride) patient suppressed, giving half-maximal suppression at 2 μg protein/ml for $VLDL_1$ and 0.1 μg protein/ml for $VLDL_2$ and $VLDL_3$ (Fig. 5). Moreover, suppressive $VLDL_1$ and $VLDL_2$ were obtained from each subject with chylomicrons in the fasting state (Types III and V) whereas the only hypertriglyceridemic VLDL preparations with normal cellular effects were from 2 patients with endogenous hypertriglyceridemia (Type IV). Consistent with the observation that delayed chylomicron clearance occurs in most, but not all, subjects with endogenous hypertriglyceridemia (22), we found that VLDL from 4 out of 6 subjects with endogenous hypertriglyceridemia were abnormally suppressive. If suppression by hypertriglyceridemic VLDL results from the presence of chylomicron remnants, the lack of suppression by $VLDL_1$ and $VLDL_2$ from normal subjects and the 2 Type IV subjects whose VLDL behaved like normal VLDL probably

TABLE II

Apoprotein Composition: Percentage Total Protein (Percentage Particle Weight)[1]

Phenotype	Subject	ApoB	ApoC	ApoE	ApoB + ApoE	Total protein
$VLDL_1$						
III	J.H.	27 (0.9)	57 (1.9)	16 (0.5)	43 (1.4)	100 (3.3)
III	M.A.	32 (0.6)	60 (1.2)	8 (0.2)	40 (0.8)	100 (2.0)
N	S.L.	16 (0.9)	76 (4.4)	8 (0.5)	24 (1.4)	100 (5.8)
N	T.T.	38 (2.4)	56 (3.6)	6 (0.4)	44 (2.8)	100 (6.4)
IV	J.B.	19 (1.1)	70 (4.0)	11 (0.6)	30 (1.7)	100 (5.7)
V	M.A.	65 (2.1)	33 (1.1)	2 (0.1)	67 (2.2)	100 (3.2)
$VLDL_2$						
III	J.H.	42 (2.4)	45 (2.6)	13 (0.7)	55 (3.1)	100 (5.7)
III	M.A.	33 (2.0)	53 (3.1)	14 (0.8)	47 (2.8)	100 (5.9)
N	S.L.	26 (1.8)	64 (4.5)	10 (0.7)	36 (2.5)	100 (7.0)
N	T.T.	32 (3.0)	58 (5.6)	10 (1.0)	42 (4.0)	100 (9.6)
IV	J.B.	31 (2.6)	58 (4.9)	11 (0.9)	42 (3.5)	100 (8.4)
V	M.A.	77 (6.6)	20 (1.7)	3 (0.3)	80 (6.9)	100 (8.6)
$VLDL_3$						
III	J.H.	58 (5.1)	29 (2.6)	13 (1.1)	71 (6.2)	100 (8.8)
III	M.A.	52 (5.6)	33 (3.6)	15 (1.6)	67 (7.2)	100 (10.8)
N	S.L.	45 (2.9)	47 (3.0)	8 (0.5)	53 (3.4)	100 (6.4)
N	T.T.	60 (6.4)	34 (3.6)	6 (0.6)	66 (7.0)	100 (10.6)
IV	J.B.	42 (5.1)	45 (5.4)	13 (1.6)	55 (6.7)	100 (12.1)
V	M.A.	89 (10.9)	9 (1.1)	2 (0.2)	91 (11.1)	100 (12.2)

[1]The total weight of each particle was taken as the sum of the amounts of free cholesterol, esterified cholesterol, triglyceride, phospholipid and total Lowry protein. Apoprotein B was determined as the difference between the total protein and the protein soluble in 4.2 M tetramethylurea (TMU). ApoC and ApoE were estimated by scanning densitometry of urea polyacrylamide gels of the TMU-soluble apoproteins.

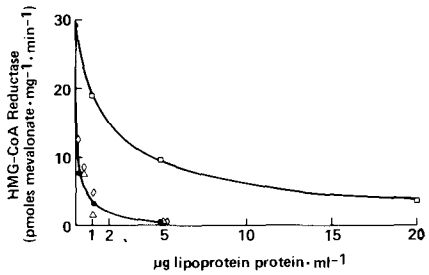

FIG. 5. Effects of Type IIb hypertriglyceridemic VLDL subclasses on HMG-CoA reductase activity in normal fibroblasts. Growth of the cells and the experimental design were the same as described in the legend to Fig. 1. Each data point is the average of duplicate determinations of HMG-CoA reductase activity in cells from duplicate dishes. The VLDL were isolated from the plasma of a subject with primary Type IIb hyperlipoproteinemia. Normal LDL (●—●); $VLDL_1$ (□—□); $VLDL_2$ (◇—◇); and $VLDL_3$ (△—△) were isolated as described in Methods.

reflect the absence of chylomicron remants in these fractions. Suppression by normal $VLDL_3$ may result in part from the presence of chylomicron remnants in this fraction. However, we have shown that VLDL remnants, produced in vitro by the action of purified lipoprotein lipase on nonsuppressive, normolipemic VLDL of S_f 60-400, have the ability to suppress HMG-CoA reductase activity in normal, but not receptor-negative, fibroblasts (5). Hence, remnants of endogenously synthesized triglyceride-rich lipoproteins produced in vivo (the smaller, S_f 20-60 fraction of VLDL) may be capable of suppression whether or not chylomicron remnants are present in the same flotation range.

In summary, these findings show that the abnormal ability of hypertriglyceridemic VLDL to suppress HMG-CoA reductase activity is present through the size spectrum of VLDL. The structural basis for the difference in cellular effects between hypertriglyceridemic VLDL and normal VLDL is not apparent from existing data. It is clear that small lipoprotein diameter alone is not necessary for suppression, since the large hypertriglyceridemic $VLDL_1$ were as effective as LDL in suppression. These studies show that there is a mechanism for direct cellular catabolism of large hypertriglyceridemic VLDL that does not involve lipoprotein lipase and is not functional for normal $VLDL_1$ or $VLDL_2$. Catabolism of VLDL by this route, presumably mediated by the LDL receptor, could account for observations that, in hypertriglyceridemia, a portion of the apoB in VLDL is cleared from the plasma compartment without first appearing in LDL (23).

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COMMUNICATIONS

The Structure of Plasma Low Density Lipoproteins: Experimental Facts and Interpretations—A Minireview[1]

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ABSTRACT

From data on size and chemical composition, low density lipoprotein (LDL) can be described as a spherical particle having cholesteryl esters and triglycerides contained in a spherical core covered by the closely packed hydrophobic ends of phospholipids and unesterified cholesterol, while the head groups of the phospholipids, together with protein, occupy the surface. Such a model is compatible with early small angle X-ray and neutron scattering studies which, by postulating spherical symmetry, assigned the LDL constituents to locations predicted from the radial electron density distribution. However, the concept of spherical symmetry, as applied to LDL structure, was recently challenged by results obtained from freeze-etching electron microscopy and small angle X-ray scattering experiments. Novel interpretations of these data suggest that the surface of LDL contains 4 electron-dense globules, located at tetrahedral positions, which have a capacity for structural remodeling at least as a function of the 2 temperatures studied (21C and 41C). It is reasonable to presume that the LDL protein (apo LDL) plays a role in the organization of the surface and overall LDL structure. However, until the chemical properties of apo LDL, and its behavior in solution and at the water-lipid interface are better understood, the validity of the proposed models cannot be assessed.

The interest in the study of the structure of the plasma lipoproteins, which by buoyant density criteria are commonly referred to as low density lipoproteins (LDL), has been recently heightened with the discovery that by interacting with specific membrane receptors, these lipoproteins exhibit regulatory functions in cell metabolism (1). Attempts to elucidate the LDL structure have been numerous and were mainly focused on the intact lipoproteins (2-4). Only recently, promising reassembly techniques have been developed and are expected to provide important new approaches to structural research (5). A fundamental limitation in the study of its structure is that LDL, like the other plasma lipoproteins, has a dynamic, fluid structure which may not be amenable to a static, rigid description. The structural flexibility of the LDL particle has been recognized (6), but the ranges of such adaptibility and the molecular events attending it have not been clarified. The assumption in any structural approach is that the definition of a basic structural pattern is compatible, both with physico-chemical data and thermodynamic principles. Once such a basic structure is understood, it should be possible to evaluate permissible structural perturbations and correlate them to LDL function(s).

On such premises, we will attempt to provide a brief overview on the field of LDL structure using old and new information.

Earlier Concepts

A dominant concept relative to the overall geometry of all plasma lipoproteins is that they are spherical or quasispherical particles having an apolar core surrounded by polar surface components (2-4). As an example, LDL_2, a lipoprotein which is separated between d 1.019 and 1.063 g/ml, is 220 Å in diameter and has a molecular weight (MW) of 2.75×10^6. The conclusion that this particle is quasispherical in shape is based on data obtained from the techniques of electron microscopy (7), small angle X-ray scattering (8-11) and analytical ultracentrifugation (12-14), but is equivocal in that none of these methods provide direct evidence of the actual shape of the LDL molecule. Electron microscopy conducted mainly on negative stained samples has important limitations since analyses were performed on dried specimens, which are conditions conducive to particle deformation (7). Similarly, the interpretation of early small angle X-ray scattering

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studies have relied on the assumption, which has been challenged by more recent ones (15) (see following text), that LDL is a particle having spherical symmetry. The ultracentrifugal studies are also in question because the hydrodynamic frictional ratio calculated from sedimentation and diffusion measurements in the analytical ultracentrifuge departs from unity, i.e., f/f_0 = 1.1, (12) a reflection of either deviation from spherical shape, particle hydration, or both. At this time, there is no way to clearly distinguish between these 2 contributions to the frictional ratio. However, LDL asymmetry, at least intended as a particle which departs from a perfect sphere, cannot be ruled out.

Attention should be given to the LDL core. Important information has been gathered from small angle X-ray (3,4,9,15,16) and neutron (17) scattering studies as well as from thermal (18) and spectroscopic (19-20) analyses. The scattering studies have been interpreted as showing that LDL has a well defined low electron density region compatible with the existence of a core containing most of the cholesteryl esters and triglycerides. A most important contribution, however, has come from the thermal analyses (18), which indicate that LDL undergoes a broad reversible thermal transition between 20 and 45 C. This cooperative transition, which is associated with the disappearance of the 36-Å fringe in the X-ray scattering curve, has been attributed to an order → disorder phase transition of the cholesteryl esters. When in the ordered phase (10 C), the cholesteryl esters have been viewed as arranged in concentric layers with a 36-Å periodicity, whereas at higher temperatures the periodicity is lost, although the radial arrangement is retained. These observations imply that some degree of organizational constraint is present, a concept which also appears to be supported by ^{13}C-NMR studies (19,20). Therefore, it would seem that, above the thermal transition, the cholesterol ring system and fatty acyl chains in LDL have a lower degree of rotational mobility compared to model systems. In this context, measurements of fluorescence depolarization using 1,6-diphenyl-1,3,5-hexatriene (DPH) and perylene have shown that the hydrophobic environments in LDL have microviscosity values which are higher than those obtained when the lipids are studied free of protein (21).

In regard to the LDL surface, current concepts confine its components, i.e., apoprotein(s), phospholipids and unesterified cholesterol, to a monolayer surrounding the apolar core. The surface location of the phospholipids receives support from kinetic studies using phospholipase A_2 as a probe (22). Although the results of these studies are compatible with an equivalent phospholipid pool, nuclear magnetic resonance (NMR) (23,24) and electron spin resonance (ESR) (25,26) data favor the existence of at least 2 distinct populations whose relative rotational motions are influenced by the extent of their interaction with proteins. Similarly unsettled is the location of unesterified cholesterol. Structural information deduced from chemical analyses has assigned this sterol to a position at the surface monolayer farther away from the aqueous environment when compared to the polar head group of phospholipids (27). Moreover, fluorescence studies have provided evidence that the unesterified cholesterol molecules of LDL are in closer proximity to the protein than to the cholesteryl esters (28). Studies using Filipin III as a probe have suggested a surface location for unesterified cholesterol although its actual position in the monolayer was not defined (R. Bittman, personal communication). Equally unsettled is the location of the LDL protein, apo B. In this case, the extent of covalent modification by succinic anhydride has been used to support the concept that this protein is predominantly located at the surface (6) but reservations against this proposal have been raised (2). The results obtained from the enzymatic digestion of LDL by proteolytic enzymes have been of little help regarding this question (2,6); the extent of hydrolysis has been limited and this may be the consequence of the intrinsic properties of the LDL protein at the LDL surface, its extent of interaction with lipids, or both. Conflicting results have also been reported on the number and nature of the peptides released after proteolysis which renders the interpretation of the results more difficult. In addition, the establishment of the secondary structure of the apoprotein at the LDL surface has not proven to be straightforward (2,5). The estimates of the relative proportion of α-helix, random coil and β-structure have varied from laboratory to laboratory and a dependence of the protein conformation on temperature and amount of lipid has been observed. Early studies support the idea of a structurally flexible apo B (29,30). The temperature dependence of the conformational changes in the apoprotein (29,30) is particularly important since it raises the question of the relationship between changes in protein conformation and lipid organization within the LDL core. Quantitative information is needed on the actual fraction of apo B exposed to the aqueous environment and that facing core lipids.

Current Concepts

It is recognized that the size and density of LDL particles vary among different normolipemic individuals and in patients with hyperlipoproteinemia (31). This microheterogeneity has been attributed to either differences in amount of lipids, particularly triglycerides (32), or to the composition of the LDL protein (33). The interest in the microheterogeneity of human LDL was heightened by the recent observations based on equilibrium density gradient ultracentrifugations (34) and on the combination of isopycnic and rate zonal density gradient ultracentrifugations (unpublished observations), indicating that the LDL class of d 1.019 to 1.063 g/ml is heterogeneous even within a single individual. Further structural studies on various LDL species should provide information of great interest.

Recently, we have extended these studies to LDL from rhesus monkeys fed a normal purina chow diet (14). The normolipidemic animals were found to contain 3 major LDL species, LDL-I, LDL-II and LDL-III, separable by a combination of isopycnic and rate zonal density gradient ultracentrifugations. Important structural differences were found, particularly between LDL-III and the other 2 LDL species. LDL-III had a mean buoyant density of 1.050 g/ml and a larger MW (3.47×10^6) than LDL-I (3.32×10^6) and LDL-II (2.75×10^6), which floated at d 1.027 and 1.036 g/ml, respectively; an apoprotein having the same amino acid composition as the other 2 LDL but a higher content in galactose and sialic acid was also found. LDL-III with the higher glycosylated apoprotein was immunologically less reactive to anti-LDL-II antisera than LDL-I and LDL-II and also exhibited spectroscopic differences by circular dichroism. Moreover, we found that LDL-III crossreacts with antisera directed to human Lp(a) lipoprotein (35). Thus, LDL microheterogeneity extends not only to size and hydrated density, but also to the type of protein moiety. This heterogeneity may be a reflection of differences in metabolic pathways and function of the various LDL species.

Recently, the concept of LDL as a centrosymmetric perfect sphere has been challenged by the studies of Luzzati and coworkers (15). A recent interpretation of their small angle X-ray scattering experiments conducted on LDL solutions at variable solvent electron densities suggests that the surface of LDL has 4 protein globules located at tetrahedral positions capable of thermally dependent structural changes. This arrangement appears to be supported by freeze-etching electron microscopic studies (36) using a novel, rapid freezing technique. Moreover, the independence of the electron density distribution of the LDL particle from that of the solvent was experimentally determined (37). Very recently, Ohtzuki et al. (38) have examined unstained preparations of human serum LDL by dark field imaging with a scanning transmission electron microscope at 2 x 10^6 magnification. Surface irregularities were noted, although no attempts were made to establish their geometry. More studies in this direction are highly desirable.

Conclusions

It is evident from the preceding discussion that the structural organization of LDL is still an unsettled question. Although one could adopt the general concept of an apolar core surrounded by a polar monolayer, this idea does not explain the molecular basis for the microheterogeneity of LDL. The structure of LDL protein has not been resolved; thus, its chemical and solution properties as well as its behavior at interfaces remain, to a large degree, unknown. Without a better knowledge of apo B, it is unlikely that the fluid-like structure of LDL and the extent of its structural flexibility to conform to lipid content and temperature can be defined. Many questions lie ahead. Among them is the establishment of the structural correlation among the various LDL species, the interrelationship between core and surface components, as well as the surface organization of the surface components. As the functional properties of LDL continue to receive attention, the need for complementary structural information will increase. This structural information, however, may not only derive from direct physico-chemical studies of the intact particles but also from the investigation of the mechanisms of their biogenesis, mode of interaction with the other plasma lipoproteins and cells and from the analyses of genetic variants. The pursuit of recent promising observations on the reassembly of LDL (5) should also prove highly informative.

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Formation of 12-[^{18}O]Oxo-*cis*-10, *cis*-15-Phytodienoic Acid from 13-[^{18}O]Hydroperoxylinolenic Acid by Hydroperoxide Cyclase

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ABSTRACT

13-[^{18}O]Hydroperoxylinolenic acid was permitted to react with an extract of flaxseed acetone powder containing hydroperoxide cyclase activity. The resulting product, 12-oxo-*cis*-10,*cis*-15-phytodienoic acid (12-oxo-PDA), contained ^{18}O in the carbonyl oxygen at carbon 12, suggesting that an epoxide was an intermediate in the hyderoperoxide cyclase reaction. A substrate specificity study showed that a *cis* double bond β,γ to the conjugated hydroperoxide group was essential for the substrate to be converted to a cyclic product by hydroperoxide cyclase.

INTRODUCTION

Polyunsaturated fatty acids with *n*-3,6,9-unsaturation can be converted to cyclic fatty acids containing a cyclopentenone ring (1) by enzymes present in a wide variety of plant tissues (2). We have previously shown that an *n*-6 hydroperoxide, formed by action of lipoxygenase, is an intermediate in the reaction sequence (3). Hydroperoxide cyclase then converts the fatty acid hydroperoxide to a cyclic fatty acid (Fig. 1). The product resulting from (9,12,15)-linolenic acid is 8-[2-(*cis*-2'-pentenyl)-3-oxo-*cis*-4-cyclopentenyl] octanoic acid, for which the common name 12-oxo-*cis*-10,*cis*-15-phytodienoic acid (12-oxo-PDA) has been proposed (1). The purpose of this investigation was to determine the origin of the carbonyl oxygen at carbon 12 of 12-oxo-PDA using ^{18}O-labeled 13-L(*S*)-hydroperoxy-*cis*-9, *cis*-15,*trans*-11-octadecatrienoic acid (13-[^{18}O]-hydroperoxylinolenic acid) as a substrate for the hydroperoxide cyclase enzyme.

EXPERIMENTAL PROCEDURES

Materials

(9,12,15)-Linolenic acid and (6,9,12)-linolenic acid were obtained from Nu-Chek-Prep, Inc., (Elysian, MN), and $^{18}O_2$ gas (> 99%) was purchased from Stohler Isotope Chemicals (Waltham, MA). Soybean lipoxygenase (21,600 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO), N,O-*bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA) from Pierce Chemical Co. (Rockford, IL), DC LSX-3-0295 silicone phase for gas chromatography from Applied Science Division (State College, PA) and precoated Anasil HF silica gel thin layer chromatography (TLC) plates from Analabs, Inc. (North Haven, CT).

Mass Spectrometry

Mass spectra were recorded with a Varian/MAT 112S GC-MS system; the glass column was 2 m x 2 mm id containing 3% DC LSX-3-0295 on 100/120 mesh Gas-Chrom Q and was temperature programmed from 165 to 220 C at 2 C/min.

Preparation of 13-[^{18}O]-Hydroperoxylinolenic Acid

Soybean lipoxygenase, which catalyzes the oxygenation of linolenic acid predominantly at carbon 13 and a minor amount at carbon 9, was used to prepare a solution that contained 13-[^{18}O]hydroperoxylinolenic acid. Water and all buffer solutions were degassed under reduced pressure, then kept under a nitrogen atmosphere prior to initiating the reaction.

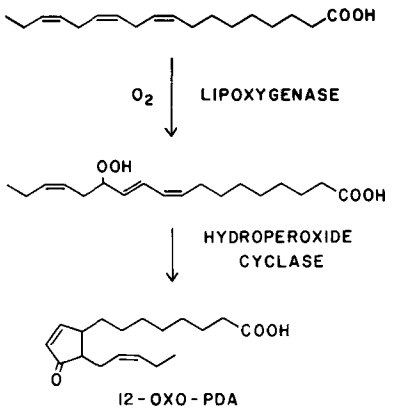

FIG. 1. Reactions catalyzed by lipoxygenase and hydroperoxide cyclase from flaxseed with (9,12,15)-linolenic acid as substrate.

(9,12,15)-Linolenic acid substrate solution (8 mM) was prepared according to the Surrey method (4) and the soybean lipoxygenase solution was prepared at a concentration of 1 mg/ml in 10 mM borate buffer (pH 9). A 16-ml test tube filled with water was placed in an inverted position in a chamber filled with water. Water was displaced from the tube with 8 ml of $^{18}O_2$; the tube was sealed with a teflon-coated septum and removed from the chamber. The buffered soybean lipoxygenase solution (1.6 ml) was added with a syringe to the remaining 8 ml of water in the tube. The oxygenation reaction was then initiated by the introduction of 0.8 ml of (9,12,15)-linolenic acid substrate solution. After 20 min, the septum was removed and the solution was adjusted from pH 9 to pH 7 with 0.2 M K-phosphate buffer (pH 6.5).

For determination of the percentage of $^{18}O_2$ incorporated into 13-hydroperoxylinolenic acid, a portion of this solution was adjusted to pH 4 and extracted with chloroform/methanol (2:1, v/v); the chloroform phase was removed, the solvent evaporated and the sample esterified with diazomethane. Hydroperoxide groups were reduced to hydroxyl groups concurrently with the saturation of double bonds by passing hydrogen through a solution of the sample dissolved in methanol with platinum oxide catalyst. The trimethylsilyloxy (OTMS) derivative of the hydroxyl group was prepared with BSTFA. Selected ion monitoring by GC-MS of the mass fragments m/e 175 vs m/e 173 $[CH_3(CH_2)_4CH(OTMS)]^+$ indicated that 94% of the hydroperoxide formed contained ^{18}O. Summation of the gas chromatographic peak areas generated by monitoring for mass fragments m/e 175 and 317 (13 isomer) and comparison with the sum of the areas generated by fragments m/e 231 and 261 (9 isomer) showed that 96% of the product was 13-hydroperoxylinolenic acid.

Formation of 12-oxo-*cis*-10, *cis*-15-Phytodienoic Acid

An enzyme solution containing hydroperoxide cyclase activity was prepared by extracting a flaxseed acetone powder (1 g) with 10 ml of 50 mM K-phosphate buffer (pH 7.0) for 30 min, then centrifuging the extract at 12,000 x g for 10 min. The enzyme solution (0.3 ml) was added to 13-[^{18}O]hydroperoxylinolenic acid solution (8.3 ml) prepared as already described. After 90 min, the pH was adjusted to 4 and the products were extracted into 10 ml of chloroform/methanol solvent (2:1, v/v). Separation of the products by TLC was accomplished with a chloroform/acetic acid solvent system (100:1, v/v) with 4 developments. The 12-oxo-PDA, which migrated just ahead of the 12,13-ketol (formed from hydroperoxide isomerase) and just behind unreacted (9,12,15)-linolenic acid, was eluted from the gel with ethyl ether, esterified with diazomethane and analyzed by gas chromatography mass spectrometry (GC-MS).

Reaction of 9-Hydroperoxy-*cis*-6, *cis*-12,*trans*-10-Octadecatrienoic Acid with Hydroperoxide Cyclase

Tomato lipoxygenase was used to prepare the 9-hydroperoxide of (6,9,12)-linolenic acid by the Matthew et al. method (5). The hydroperoxide product was purified by TLC (hexane/ethyl ether/acetic acid, 65:35:1, v/v), then eluted from the gel with ethyl ether. The solvent was evaporated and the sample was redissolved in 95% ethanol (0.2 ml). A small portion of this preparation (ca. 0.2 μmol) was esterified and analyzed as the reduced, saturated, trimethylsilyloxy derivative by summing ions m/e 173 plus 315 (13 isomer) and m/e 229 plus 259 (9 isomer) by GC-MS as already described. The results indicated that 81% of the product was the 9-hydroperoxy isomer, thus assuring that the desired product, 9-hydroperoxy-*cis*-6,*cis*-12,*trans*-10-octadecatrienoic acid [9-hydroperoxy-(6,10,12)-linolenic acid], had been obtained.

The 9-hydroperoxy-(6,10,12)-linolenic acid was reacted with an extract of flaxseed acetone powder containing hydroperoxide cyclase activity. The ethanolic solution of the compound (ca. 4 μmol) was added to 20 ml of 50 mM K-phosphate buffer (pH 7.0) and 1 ml of flaxseed acetone powder extract (1 g in 10 ml of 50 mM K-phosphate buffer, pH 7) was added. After 1 hr, the reaction mixture was adjusted to pH 4 and the products were extracted with 35 ml of chloroform/methanol (2:1, v/v). Separation of the products was done using TLC with chloroform/acetic acid solvent (100:1, v/v) with 3 developments. Products were visualized by exposing a portion of the plate to iodine vapor, then eluted from the gel, esterified with diazomethane and analyzed by GC-MS.

RESULTS AND DISCUSSION

Figure 2 shows the mass spectrum of 12-[^{18}O]oxo-PDA formed enzymically from 13-[^{18}O]hydroperoxylinolenic acid. The molecular ion at m/e 308 and the mass fragments at m/e 277 $[M\text{-}OCH_3]^+$, m/e 240 $[M\text{-}(C_5H_9) + H]^+$, m/e 179 $[M\text{-}(CH_2)_5COOCH_3]^+$ and m/e 165 $[M\text{-}(CH_2)_6COOCH_3]^+$ were 2 daltons

higher than the corresponding masses obtained when 13-[^{16}O]hydroperoxylinolenic acid was the substrate (m/e 306, 275, 238, 177 and 163). Comparison of these ^{18}O fragments in Figure 2 with the intensities of the corresponding ^{16}O fragments indicated that the compound contained 90% ^{18}O in the oxo group compared to 94% in the hydroperoxide. This small apparent decrease in ^{18}O enrichment was not regarded as experimentally significant. Thus, the mass spectra indicated that 13-hydroperoxylinolenic acid was converted to 12-oxo-PDA with nearly complete retention of ^{18}O in the carbonyl oxygen at carbon 12. This result is similar to that reported for the synthesis of 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid (α-ketol) from 13-hydroperoxylinoleic acid, catalyzed by hydroperoxide isomerase. This enzyme from flaxseed (6) and corn germ (7) has been shown to catalyze the formation of the α-ketol with retention of ^{18}O in the 12-oxo group, but with ^{16}O in the 13-hydroxy group, presumably from water.

Gardner has recently suggested a mechanism for hydroperoxide isomerase action based on the incorporation of ^{18}O into the 12-oxo group and on work in his own laboratory, which showed that substitution by nucleophiles other than water could occur at the hydroperoxide carbon atom with inversion of stereoconfiguration from *S* to *R* (8). He proposed the formation of an epoxy-cation intermediate by loss of OH^- from the hydroperoxide group. The intermediate could react with a nucleophile (OH^-) in a bimolecular nucleophilic substitution (S_N2) reaction at the carbon originally bearing the hydroperoxide group. This mechanism accounted for the transfer of a hydroperoxide oxygen to a vicinal carbon and the inversion of configuration at the hydroperoxide carbon.

A similar mechanism involving an epoxy-cation intermediate appears likely for the hydroperoxide cyclase reaction (Fig. 3). Abstraction of a proton from carbon 12 by the enzyme could lead to an enolate anion at carbons 12 and 13; rearrangement of this intermediate would give cyclization between carbons 9 and 13. 13-Hydroperoxylinoleic acid, which differs from 13-hydroperoxylinolenic acid only by the absence of unsaturation at carbon 15, is unreactive with hydroperoxide cyclase (3) (Fig. 4A). However, when 9-hydroperoxy-(6,10,12)-linolenic acid was allowed to react with a flaxseed extract contain-

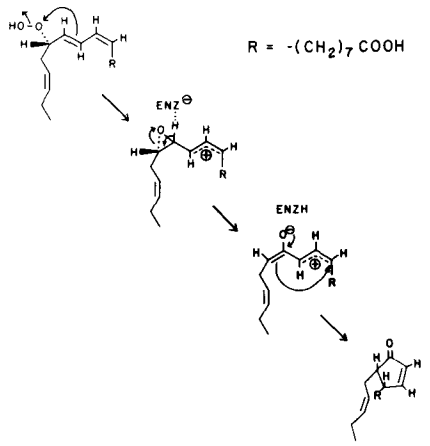

FIG. 3. Proposed mechanism for the cyclization of 13-hydroperoxylinolenic acid by the hydroperoxide cyclase enzyme.

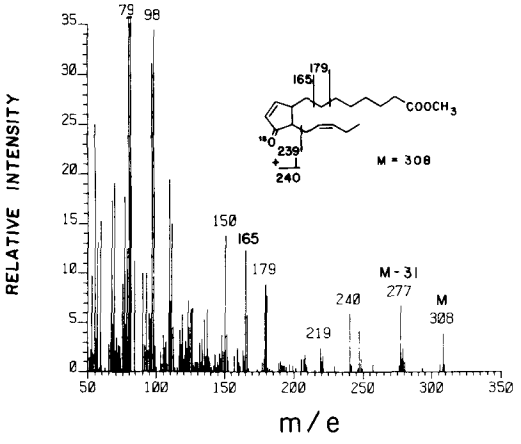

FIG. 2. Mass spectrum of 12-[^{18}O]oxo-*cis*-10,*cis*-15-phytodienoic acid (12-oxo-PDA) resulting from the reaction of 13-[^{18}O]hydroperoxylinolenic acid with an extract of flaxseed acetone powder containing hydroperoxide cyclase activity.

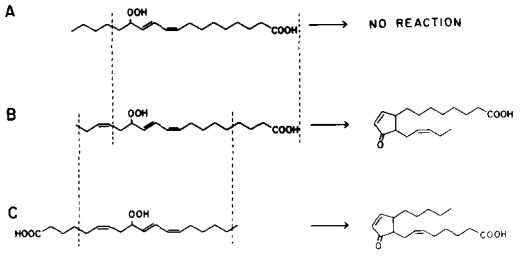

FIG. 4. Reactions showing the products of hydroperoxide cyclase activity from (A) 13-hydroperoxylinoleic acid, (B) 13-hydroperoxylinolenic acid, and (C) 9-hydroperoxy-(6,10,12)-linolenic acid. Dashed lines indicate portions of molecules with identical structure. Experiments showed that only 13-hydroperoxylinolenic acid (B) and 9-hydroperoxy-(6,10,12)-linolenic acid (C) were reactive with hydroperoxide cyclase.

ing hydroperoxide cyclase activity, a cyclic compound was identified as a product. Over a range of 13 carbons, this substrate had the same chemical structure as 13-hydroperoxy-linolenic acid. The cyclic product of this hydroperoxide cyclase reaction was proposed to be 8-(2-oxo-5-pentyl-*cis*-3-cyclopentenyl)-*cis*-6-octanoic acid on the basis of its mass spectrum, which showed ions at m/e 306 $[M]^+$, m/e 275 $[M\text{-}OCH_3]^+$, and m/e 152 $[C_5H_4O(CH_2)_4CH_3+H]^+$. Figure 4C shows the structure of the hydroperoxide substrate and the proposed structure of the cyclic product.

In a previous paper, we reported that *n*-3 unsaturation in the fatty acid was necessary for recognition by the hydroperoxide cyclase enzyme (3). However, the results reported here demonstrated that a *cis* double bond β,γ to the conjugated hydroperoxide group was the essential feature. Apparently, the carboxyl group was not a factor in the attachment of the substrate hydroperoxide to the enzyme. It is likely that the substrate can attach to the active site with the carboxyl group in either direction. The important factor is that the substrate molecule for hydroperoxide cyclase must have a 4(*S*)-hydroperoperoxy-*cis*-1,*cis*-7,*trans*-5-octatriene group.

ACKNOWLEDGMENTS

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15-Hydroperoxyeicosatetraenoic Acid Inhibits Human Platelet Aggregation

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ABSTRACT

Using human platelets isolated from their plasma, we showed that 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) inhibits platelet aggregation induced either by arachidonic acid or prostaglandin H_2 analog. 15-HPETE does not modify platelet prostaglandin and thromboxane formation from exogenous arachidonic acid but does decrease platelet lipoxygenase activity.

INTRODUCTION

Endothelial cells generate a very potent inhibitor of platelet aggregation called prostacyclin (1,2). This compound is produced from arachidonic acid via prostaglandin endoperoxides (3). Prostacyclin synthetase is strongly inhibited by various hydroperoxides (4) and chiefly by 15-hydroperoxy-5,8,11,13-eicosatetranoic acid (15-HPETE) (5).

Prostaglandins are synthesized during platelet aggregation (6). More recently, it was shown that platelets produce thromboxane A_2, a potent aggregating agent, from arachidonic acid (7).

Platelet lipoxygenase provides 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) which inhibits platelet thromboxane synthetase (8). In this work, the effect of 15-HPETE on platelet aggregation and arachidonic acid metabolism was investigated.

MATERIALS AND METHODS

Reagents

Arachidonic acid and soybean lipoxidase were obtained from Sigma, St. Louis, MO. [1-^{14}C] Arachidonic acid (> 50 Ci/mol) from the Radiochemical Centre of Amersham was used. Standard prostaglandins and analogs were generous gifts from Dr. J.E. Pike of the Upjohn Company, Kalamazoo, MI. Organic solvents were products of Prolabo, Paris, France.

NADPH and GSH reductase were purchased from Boehringer, Mannheim, West Germany. Partially purified GSH peroxidase was prepared using a previously described method (9). 15-HPETE was synthesized from arachidonic acid with a technique previously described (10) with 0.1 M borate buffer, pH 9 (11).

Purification and Measurement of 15-HPETE

Biosynthesized 15-HPETE was purified by thin layer chromatography (TLC) on silica gel plates with hexane/diethyl ether/acetic acid (60:40:1) as eluent. To localize 15-HPETE with a radioscanner, a low specific radioactivity of its precursor (0.5 mCi/mol) was used. A spot of 15-HPETE was extracted with methanol and the compound assayed by its absorbance at 234 nm. A more specific assay was performed using an enzymic method (9).

Incubation Studies

Human platelets were isolated from their plasma as previously described (12). Platelet aggregation (0.3 x 10^9 platelets/ml) was performed according to Born's turbidimetric method (13). 15-HPETE or its solvent (less than 1/200 of ethanol) was simultaneously added with aggregating agents.

Metabolism of exogenous arachidonic acid by human platelets was studied in the presence or absence of 15-HPETE. Incubations were done as already described with sodium arachidonate (0.5 Ci/mol) 10^{-5} M for 4 min at 37 C.

Prostaglandins and related compounds were extracted, purified and quantified using a radiochemical technique (14). Because of the low specific radioactivity of 15-HPETE, interferences did not disrupt the radiochemical technique.

RESULTS

Platelet aggregation induced by sodium arachidonate was inhibited by 15-HPETE. The aggregations induced by sodium arachidonate 10^{-5} M and $0.25.10^{-5}$ M were abolished by 2.10^{-5} M and $0.8.10^{-5}$ M, respectively of 15-HPETE. IC_{50} was obtained by half concentrations of the inhibition (Fig. 1).

The effect of the 9-methano analog of PGH_2 on platelets pretreated with aspirin also was counteracted by 10^{-5} M of 15-HPETE (Fig. 2). Moreover, platelet aggregation induced by PGH_2 was inhibited by 15-HPETE in similar

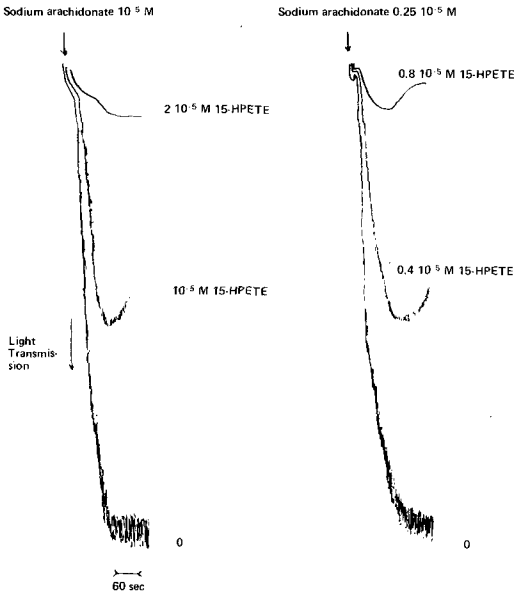

FIG. 1. Effect of 15-HPETE on arachidonate-induced platelet aggregation. 15-HPETE and arachidonate were added to platelets at the same time. These results were observed five times.

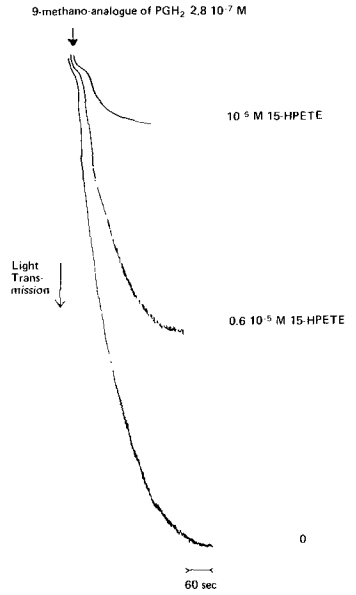

FIG. 2. Effect of 15-HPETE on 9-methano analog of PGH_2-induced platelet aggregation. Platelets were pretreated with aspirin. These results were observed five times.

conditions (results not shown).

The effect of 15-HPETE 2.10^{-5} M was tested on exogenous arachidonic acid use by platelets. The main stable metabolites from arachidonic acid produced by platelet prostaglandin synthetase and lipoxygenase were determined and results are shown in Table I. Only 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) provided by lipoxygenase pathway was decreased in the presence of 15-HPETE. Synthesis of other metabolites from prostaglandin and thromboxane synthetase pathways was not changed.

DISCUSSION

The inhibition of human platelet aggregation by hydroperoxides was previously reported for linoleic acid hydroperoxide (15). However, the earlier investigators used 10-fold higher linoleic acid hydroperoxide combination to block aggregation.

Inhibition of platelet aggregation observed in our experiments suggest that prostaglandin biosynthesis is not affected because either arachidonic acid or PGH_2 analog-induced aggregation were abolished by about the same concentrations of 15-HPETE. In addition, prostaglandins and thromboxane synthesized from exogenous arachidonate were not modified by the hydroperoxide.

Thus, platelet inhibition by 15-HPETE is not explainable by its action on cyclooxygenase and/or thromboxane synthetase, as previously mentioned (16). The inhibition of the formation of lipoxygenase products could result from structural analogy between these products and 15-HPETE.

TABLE I

Production of Prostaglandins and Related Compounds by Human Platelets from Exogenous Sodium Arachidonate (10^{-5} M) (n = 12). Statistic t-Test Was Used

nmol/10^9 platelets	$PGF_{2\alpha}$	PGE_2	TXB_2	HHT	12-HETE
Control	0.26 ± 0.12	0.38 ± 0.18	3.5 ± 2.1	2.4 ± 2.1	4.6 ± 2.6
15-HPETE	0.28 ± 0.11	0.38 ± 0.16	2.5 ± 1.2	1.9 ± 1.1	2.8 ± 1.4
2 x 10^{-5} M	NS	NS	NS	NS	$P < 0.05$

Some investigators have shown that inhibition of platelet lipoxygenase induces a decreased aggregation (17). However, the inhibition of platelet lipoxygenase by 15-HPETE we observed seems insufficient to explain the abolition of aggregation. Besides, platelet aggregation induced by thrombin, an agent which does not need prostaglandin cyclic endoperoxides and thromboxane A_2 to aggregate platelets, is not inhibited by 15-HPETE (results not shown). The effect of 15-HPETE on platelet aggregation could instead be explained by counteracting the action of these pro-aggregatory molecules.

15-HPETE was previously shown to inhibit prostacyclin synthetase (4,5) and the IC_{50} observed in these experiments was ca. 10^{-6} M. Our results showed a higher IC_{50} of platelet aggregation. Thus, in the presence of both endothelial cells and platelets, 15-HPETE should be a more specific inhibitor of prostacyclin formation.

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Alkenyl and Alkyl Ether Phospholipids in Pig Mesenteric Lymph Node Lymphocytes[1]

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ABSTRACT

Significant amounts of alkenyl and alkyl ether phosphoglycerides were found in pig mesenteric lymph node lymphocytes. The choline phosphoglycerides are composed of 2.6% alkenyl ether and 25.7% alkyl ether compounds besides 71.7% diacyl analogs. The ethanolamine phosphoglycerides consist of 50.2% alkenyl ether, 7.8% alkyl ether and 42.0% diacyl compounds. The fatty chain compositions at the 1- and 2-positions of each lipid class were analyzed.

INTRODUCTION

The composition and metabolism of phospholipids in lymphocytes has been studied by several investigators (1-7) and the role of phospholipids during lymphocyte activation has also been discussed. It has been demonstrated that there is a rapid increase in the activity of lysolecithin acyltransferase with subsequent changes in the acyl moieties of phospholipids after stimulating the lymphocytes with mitogens (3,6). However, these studies were generally carried out on mixtures of diacyl and ether-containing phosphoglycerides (plasmalogen and 1-alkyl-2-acyl-GPC or GPE) and data on individual phosphoglycerides are not available. It is necessary to study ether-containing phosphoglycerides in order to clarify the role of the phospholipids in lymphocytes, since they are so different from diacyl compounds in several biochemical properties, e.g., in biosynthetic routes (8), fatty chain composition (9), turnover rates (10,11) and in substrate specificities for enzymes (8,12).

This investigation shows that high levels of ether-containing phosphoglycerides (which account for ca. 25% of total phospholipids) are found both in choline and ethanolamine fractions of lymphocytes and that their fatty chain compositions are considerably different from diacyl phosphoglycerides.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and solvents were distilled before use.

Preparation of Lymphocytes

Pig lymphocytes were prepared by a modification of the Allan and Michell method (13). Mesenteric lymph nodes were removed from young pigs (average age 6 mo) and were placed immediately into ice-cold Eagle's medium. The lymph nodes were freed from surrounding tissues, cut into small pieces and dispersed to yield lymphocytes. The resulting dispersion was filtered through a cotton wool plug and centrifuged. The sedimented cells were treated with 0.83% NH_4Cl-Tris-HCl buffer (pH 7.4) to remove erythrocytes and washed 3 times with medium. The final preparation contained mainly small lymphocytes (>98%) as assessed by microscopic examination and the cell viability was 80-90% by Trypan blue dye exclusion test.

Extraction and Fractionation of Lipids

The lipids were extracted as described by Bligh and Dyer (14). Choline and ethanolamine fractions were isolated as described previously (9). Each fraction gave a single spot on a precoated silica plate (Merck, Darmstadt). The purity of the individual phosphoglycerides was also checked by the Dawson method (15). Throughout the fractionation procedure, a small amount of butylated hydroxytoluene was added to the solvents.

Separation and Quantitative Analyses of Alkenyl, Alkyl and Diacyl GPC or GPE

1-Radyl-2-acyl-3-acetylglycerol was prepared from both choline and ethanolamine phosphoglycerides as described previously (9). Three types of diradyl acetates, 1-alkenyl-2-acyl-3-acetylglycerol, 1-alkyl-2-acyl-3-acetylglycerol and 1,2-diacyl-3-acetylglycerol, were separated by thin layer chromatography (TLC) according to the Renkonen and Luukkonen method (16). The quantities of the fatty acyl moieties of each lipid class were estimated by gas liquid chromatography (GLC), using 17:0 methyl ester as an internal standard. The occurrence of alkenylacyl, alkylacyl and diacyl phosphoglycerides was confirmed by mild alkaline and

[1]Abbreviations: Fatty chains are designated by number of carbon atoms:number of double bonds, e.g., 18:1 for oleic acid; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine.

acid hydrolysis of choline and ethanolamine fractions according to Dawson (15).

Determination of Fatty Acid Distribution

Fatty acyl residues of the lipids were analyzed as the methyl esters by GLC (9). To investigate the positional distribution of fatty acids in 1,2-diacyl-3-acetylglycerol, fatty acids at the 1-position were liberated by *Rhizopus delemar* lipase (Seikagaku Kogyo Co.) and the resulting monoglycerides were separated by TLC and transmethylated as described previously (10). Snake venom phospholipase A_2 *(Naja naja atra)* was also used to remove the fatty acid residues from the 2-position of choline and ethanolamine phosphoglycerides (9). The resulting lyso-compounds were separated by TLC and transmethylated. The positional distribution of fatty acids obtained by these 2 methods showed similar patterns.

Determination of Alkenyl and Alkyl Chain Distribution

1-Alkenyl-2-acyl-3-acetylglycerol was treated with acetic acid containing 1% $HgCl_2$ and the aldehydes liberated were determined as described earlier (9). Trimethylsilyl derivatives of 1-alkylglycerol were prepared from 1-alkyl-2-acyl-3-acetylglycerol and analyzed by GLC (10).

RESULTS AND DISCUSSION

Choline and ethanolamine phosphoglycerides were found to be the predominant components, accounting for 45% and 24% of the total phospholipids, respectively, as determined by 2-dimensional TLC (17).

1-Radyl-2-acyl-3-acetylglycerol derivatives of choline and ethanolamime phosphoglycerides from pig lymphocytes were separated by TLC as shown in Figure 1. Quantities of each type of 1-radyl-2-acyl-3-acetylglycerol were determined

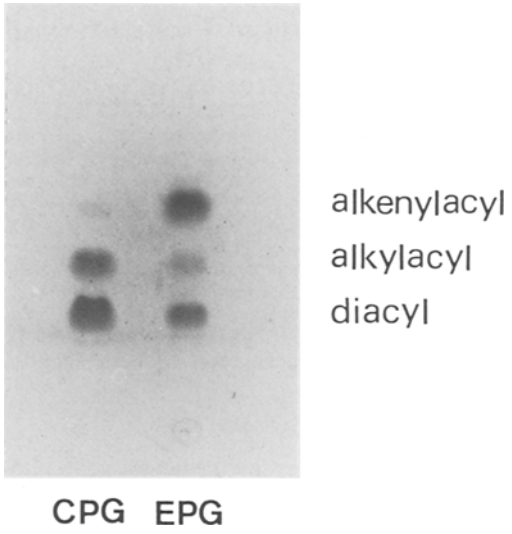

FIG. 1. TLC separation of 1-alkenyl-2-acyl-3-acetylglycerol, 1-alkyl-2-acyl-3-acetylglycerol and 1,2-diacyl-3-acetylglycerol derivatives of choline and ethanolamine phosphoglycerides (CPG and EPG) from pig lymphocytes. The plate was developed with petroleum ether/ethyl ether/acetic acid (90:10:1) and then with toluene. The spots were visualized with 50% sulfuric acid and charring.

by GLC assays of amounts of fatty acyl moieties in each lipid class (Table I, exp. 1). The amounts of alkenylacyl, alkylacyl and diacyl compounds were also estimated by mild alkaline and acid hydrolysis of the phosphoglycerides (Table II, exp. 2). The choline fraction contains a considerable amount of alkyl ether compounds (25.7%), whereas the portion of alkenyl ether compounds is only small (2.6%). In contrast, the ethanolamine fraction contains a significant amount of alkenyl ether compounds (50.2%) and a relatively small amount of alkyl ether compounds (7.8%). In an earlier study, it was reported that the spleen is also

TABLE I

Class Composition of Choline and Ethanolamine Phosphoglycerides from Pig Mesenteric Lymph Node Lymphocytes[a]

	Choline phosphoglycerides (mol %)		Ethanolamine phosphoglycerides (mol %)	
Class	Exp. 1[b]	Exp. 2[c]	Exp. 1	Exp. 2
Alkenylacyl	2.6 ± 0.6	2.2 ± 1.0	50.2 ± 3.3	49.9 ± 3.6
Alkylacyl	25.7 ± 2.8	23.2 ± 3.6	7.8 ± 1.0	7.9 ± 1.1
Diacyl	71.7 ± 3.1	74.6 ± 3.8	42.0 ± 3.7	42.2 ± 2.9

[a]The mean percentages ± SD were taken from different samples.

[b]Values were obtained from the quantities of the fatty acyl moieties of each lipid class in exp. 1. (n=6).

[c]Values were obtained by successive hydrolysis of the phosphoglycerides in exp. 2. (n=3).

TABLE II

Fatty Chain Composition of Choline Phosphoglycerides (mol %)[a]

Fatty chain	Class	Alkenylacyl		Alkylacyl		Diacyl	
	Position	1[b]	2[c]	1[d]	2	1	2
16:0		64.0 ± 0.8	28.2 ± 2.8	29.4 ± 1.0	35.2 ± 3.5	45.3 ± 1.6	34.0 ± 2.6
16:1		---	5.0 ± 1.2	---	3.1 ± 0.8	4.0 ± 1.0	3.5 ± 0.9
18:0		21.4 ± 1.2	5.1 ± 1.7	13.8 ± 1.7	1.4 ± 0.5	19.0 ± 1.7	0.5 ± 1.0
18:1		14.6 ± 1.6	13.7 ± 3.9	56.8 ± 1.9	6.7 ± 1.2	25.2 ± 2.8	20.2 ± 2.9
18:2		---	7.2 ± 1.8	---	7.0 ± 1.4	4.8 ± 0.9	15.0 ± 3.7
18:3 + 20:1		---	1.6 ± 0.6	---	0.3 ± 0.2	1.7 ± 0.7	0.3 ± 0.2
20:3		---	2.2 ± 1.4	---	3.1 ± 1.1	---	3.4 ± 0.2
20:4		---	29.6 ± 3.6	---	32.6 ± 3.1	---	18.7 ± 0.7
22:4		---	5.3 ± 1.4	---	5.3 ± 1.2	---	1.8 ± 0.3
22:5		---	1.3 ± 0.3	---	3.0 ± 1.2	---	1.2 ± 0.3
22:6		---	0.8 ± 0.4	---	2.3 ± 1.3	---	1.4 ± 0.3

[a] The mean percentages ± SD were taken from different samples (n=5).
[b] Alkenyl glyceryl ethers were hydrolyzed to liberate aldehydes and analyzed by GLC.
[c] Fatty acids were analyzed as the methyl esters.
[d] Alkyl glyceryl ethers were analyzed as the trimethylsilyl derivatives.

rich in alkyl ether compounds (18). It may be possible that lymphoid tissues generally contain high amounts of alkyl ether compounds.

The fatty chain composition of choline phosphoglycerides is shown in Table II. The fatty chain at the 1-position of each lipid class is mainly composed of 16:0, 18:0 and 18:1; however, the distribution among the choline phosphoglycerides is considerably different. Most striking is the high percentage of 18:1 (56.8%) at the 1-position in alkyl ether compounds, though a considerable amount of 18:1 (25.2%) is also found at the 1-position in diacyl compounds. The fatty chain at the 1-position of alkenyl ether compounds is most highly saturated. The fatty chain at the 2-position is relatively similar in each lipid class, although the proportion of 20:4 is smaller in the diacyl compounds than in ether-linked compounds. A significant amount of 16:0 is observed at the 2-position in each lipid class.

The fatty chain distribution of ethanolamine phosphoglyceride is shown in Table III. The distribution of the fatty chains at the 1-position in each lipid class are again quite different from each other. The highest amount of 16:0 (43.8%) is located in alkenyl ether compounds. On the other hand, almost half of the fatty chains at the 1-position are accounted for by 18:1 in alkyl ether compounds and by 18:0 in diacyl compounds. The fatty chain composition at the 2-position shows a similar pattern both in alkenyl ether and diacyl compounds. The main constituent is 20:4 (46.9% and 56.2%) and other fatty acids between 16:0 and 22:6 are widely distributed. In alkyl ether compounds, a higher amount of 16:0 (18.0%) and 22:4 (22.5%) are observed at the 2-position in place of a relatively small amount of 20:4 (24.9%).

The fatty chain composition of diacyl compounds obtained in this investigation is in general agreement with the results of an earlier study (6). It has been reported that lymphocytes contain a considerable amount of 16:0 at the 2-position and 18:1 at the 1-position in choline phosphoglycerides (6). Similar results were observed, not only in diacyl compounds but also in alkyl ether compounds in this study, although in some cases only a small amount of 16:0 could be observed at the 2-position in diacyl compounds. This unusual distribution of fatty chains is also observed in tumor cells (19). The fatty chain compositions of ether-containing phosphoglycerides in lymphocytes show marked differences compared with those in other tissues (9-11,20).

Although ether-containing phosphoglycerides are widely distributed in animal tissues,

TABLE III

Fatty Chain Composition of Ethanolamine Phosphoglycerides (mol %)[a]

Fatty chain	Class	Alkenylacyl		Alkylacyl		Diacyl	
	Position	1[b]	2	1	2	1	2
16:0		43.8 ± 1.0	4.6 ± 0.3	33.9 ± 0.8	18.0 ± 1.7	16.3 ± 2.8	2.9 ± 1.3
16:1		---	0.8 ± 0.1	---	1.4 ± 0.4	1.2 ± 0.4	0.6 ± 0.3
18:0		38.8 ± 0.8	0.8 ± 0.3	17.4 ± 0.8	1.2 ± 0.5	51.9 ± 8.6	1.1 ± 1.7
18:1		17.4 ± 1.2	5.6 ± 0.6	48.7 ± 1.6	6.1 ± 1.1	25.0 ± 3.9	11.4 ± 2.3
18:2		---	2.6 ± 0.3	---	2.7 ± 0.6	5.1 ± 1.1	4.7 ± 2.9
18:3 + 20:1		---	0.5 ± 0.6	---	0.5 ± 0.1	0.5 ± 0.4	0.9 ± 0.4
20:3		---	4.4 ± 1.8	---	4.4 ± 1.6	---	4.0 ± 0.9
20:4		---	46.9 ± 3.5	---	24.9 ± 2.3	---	56.2 ± 2.6
22:4		---	16.0 ± 1.8	---	22.5 ± 2.0	---	7.9 ± 1.4
22:5		---	10.6 ± 2.2	---	12.9 ± 3.1	---	5.3 ± 1.0
22:6		---	7.2 ± 1.9	---	5.4 ± 1.8	---	5.0 ± 1.0

[a]The mean percentages ± SD were taken from different samples (n=5).
[b]Analytical procedures were as in Table II.

their biological role is yet unclear. Recently, it has been shown that arachidonic acid is transferred to ethanolamine plasmalogen from other phospholipids in platelets exposed to thrombin (21). Furthermore, Demopoulos et al. (22) reported that 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine has a potent biological activity to platelets and this compound is apparently identical to the native platelet-activating factor (PAF) derived from basophils. It is possible that ether-containing phosphoglycerides play an important role also in lymphocytes in the course of immunological processes. In further investigations, we hope to compare in detail the ether-containing phosphoglycerides of mitogen-stimulated lymphocytes with resting cells.

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METHODS

Synthesis of α-Hydroxy Stearyl Coenzyme A

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ABSTRACT

Synthesis of α-hydroxy stearyl CoA from N-hydroxysuccinimide ester of a α-hydroxy stearic acid and coenzyme A is reported. The CoA derivative has been isolated, purified and characterized from its spectral data and chemical properties.

INTRODUCTION

White matter of the adult brain contains a considerable proportion of α-hydroxy fatty acids (1,2). The very long chain α-hydroxy fatty acids are unique components of both cerebrosides and sulfatides and comprise more than one-half of the total cerebroside fatty acids (3). The hydroxy fatty acids are formed from the corresponding nonhydroxy fatty acids (4) and are oxidized to nonhydroxy fatty acids containing one less carbon atom (5). It is unknown whether the hydroxy fatty acids also undergo elongation like the nonhydroxy fatty acids (6). We have been interested in studying the metabolic fate of hydroxy fatty acids in brain using synthetically prepared coenzyme A ester of these fatty acids.

The synthesis of various S-acyl CoA has been achieved by acid chloride, anhydride, thiol ester exchange and N-hydroxy succinimide ester methods (7-14). Although acid chloride and anhydride methods work well for the synthesis of S-acyl CoA from mono- and dicarboxylic fatty acids, they present difficulties when competitive groups such as hydroxy or amino are present on the aliphatic chain of the fatty acid or when polymeric anhydrides are formed, as in the case of pimelic acid (12). Besides, there are problems in the preparation of such acylating agents since reagents such as thionyl chloride, phosphoryl chloride, oxalyl chloride or ethyl chloroformate react not only with the acid functionality but also with the hydroxy group thereby leading to undesirable products.

N-Hydroxysuccinimide in the presence of equivalent amounts of dicyclohexylcarbodiimide and hydroxy fatty acid at room temperature forms N-hydroxy succinimide ester of the hydroxy fatty acid as the sole product because of the preferred reaction at the carboxylic group. The ester, upon treatment with coenzyme A, yields the desired product in quantitative yield.

MATERIALS AND METHODS

α-Hydroxystearic acid, technical grade, was purchased from ICN Pharmaceuticals, Inc., Plainview, NY. It was crystallized twice from methanol before use. Thioglycolic acid, N-hydroxysuccinimide and coenzyme A (lithium salt, 85%) were obtained from Sigma Chemical Company, St. Louis, MO. Various organic solvents, e.g., ethyl acetate, acetone, diethyl-ether and tetrahydrofuran, were purchased from Fischer Scientific Company, Fair Lawn, NY, and were 99.9 mol % pure and used without further purification. Dicyclohexyl-carbodiimide was purchased from Eastman Kodak Company, Rochester, NY. Tetrahydro-furan was freshly distilled from lithium aluminium hydride before use to eliminate any contamination of peroxides. The infrared (IR) spectra were obtained on a Perkin-Elmer model 337 Spectrophotometer as Nujol mulls and ultraviolet (UV) on a Unicam SP 800 B Spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian model 56/60 A Spectrometer in deuterio-chloroform, using tetramethylsilane as an internal standard. Mass spectra were obtained on a Finnegan 1015 quadruple mass spectrometer. Melting points were determined on a precalibrated "Thermopan" apparatus. Thin layer chromatography (TLC) was performed on 0.1-mm thick silica gel plates purchased from Eastman Kodak Company, Rochester, NY.

Preparation of N-Hydroxysuccinimide Ester of α-Hydroxy Stearic Acid

N-Hydroxysuccinimide (1.15 g) and α-hydroxystearic acid (3.0 g) were dissolved in dry ethyl acetate (600 ml) with slight warming. The solution was brought to room temperature and dicyclohexylcarbodiimide (2.06 g) in 75 ml of dry ethyl acetate added. The reaction mixture was stirred at room temperature overnight. Dicyclohexylurea precipitated during

the reaction was removed by filtration; the filtrate was stripped off the organic solvent on a rotary evaporator. The residue was crystallized twice from methanol to give 3 g of the product, mp 86-87 C. It showed a single spot on TLC plate in chloroform or chloroform containing 0.2% methanol and was characterized as follows: IR: 3500 cm^{-1}, OH; 1818 cm^{-1}, ester CO; 1786 and 1724 cm^{-1}, ring CO; NMR: δ 8.85, singlet, heteroring methylenes; the rest of the absorptions from δ 2.20-1.0 result from aliphatic protons. The mass spectrum did not show the molecular ion. The fragment at m/e 283 corresponds to the loss of N-hydroxy succinimide, consistent with our previous findings (14).

Preparation of α-Hydroxy Stearyl CoA

N-Hydroxysuccinimide ester of α-hydroxy stearic acid (794 mg) thioglycolic acid (46 mg) and coenzyme A (50 mg) were dissolved in tetrahydrofuran/water mixture (2:1, 75 ml) and the solution stirred under nitrogen at room temperature. 1 N sodium hydroxide solution (2 ml) was added slowly so that the pH of the solution did not exceed 8.0. The reaction was complete in 4 hr as shown by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) test. Tetrahydrofuran was removed from the reaction mixture under reduced pressure at room temperature and to the remainder aqueous cold solution was added 6 ml of 10% perchloric acid to precipitate any unreacted N-hydroxysuccinimide ester, α-hydroxy stearyl-S-thioglycolic acid and α-hydroxy stearyl CoA. The solution was filtered and the residue washed with 0.8% perchloric acid (20 ml) followed by peroxide free diethylether (5 x 30 ml) and acetone (3 x 10 ml) to remove any α-hydroxy stearic acid, α-hydroxy-S-thioglycolic acid and N-hydroxysuccinimide ester. The residue was taken in 15 ml water and the pH of the solution brought to 5.5 with sodium bicarbonate. the solution was filtered and the residue washed with an additional 10 ml of water. the combined filtrate was treated with 10% perchloric acid (3 ml) to precipitate α-hydroxy stearyl CoA. The solution was centrifuged and the residue washed with 0.8% perchloric acid (5 ml), acetone (2 x 5 ml) and finally diethylether (3 x 5 ml). α-Hydroxy stearyl CoA was finally dried under nitrogen and weighed, 60 mg (87%).

Characterization

α-Hydroxy stearyl CoA did not show the DTNB test for the free thiol group. This test, however, became positive upon hydrolysis with alcoholic potassium hydroxide. The UV spectrum of α-hydroxystearyl CoA showed a thioester peak at 232 nm when measured against a reference solution of coenzyme A. This peak disappeared upon treatment with alcoholic potassium hydroxide. An assay of the coenzyme ester from its hydroxmate derivative formation indicated it to be 90% pure.

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A Simple Chromatographic Method for Purification of Egg Lecithin

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ABSTRACT

Egg lecithin was purified from the $CdCl_2$-lecithin complex by column chromatography on Alumina. The yield from 5 eggs was 2.8 g. The purified lecithin had correct chemical values for pure lecithin and a fatty acid composition similar to lecithin prepared by other methods. The method probably can be adapted for purification of other lipids containing the phosphocholine moiety and for purification of synthetic lecithin.

INTRODUCTION

In the most frequently used screening tests for syphilis, antibodies are demonstrated in blood and cerebrospinal fluid after binding to an antigen consisting of lecithin, cardiolipin and cholesterol. Our laboratory has for at least 20 years used the Pangborn method (1) for producing egg lecithin (hen) for use in the antigen. Experience has shown that new lots of lecithin constantly cause difficult standardization of the antigen. The difficulties result from differences in purity and content of fatty acids in different lots of lecithin. To obtain the standardized antigen it is essential to use some simple preparation methods which are easily reproduced and give pure lecithin with no trace of lysolecithin.

Most methods for preparation of lecithin from egg yolks are based on the work of Pangborn (1), in which lecithin is purified as the $CdCl_2$-lecithin complex (CLC) and then transformed into lecithin. This transformation, which consists of washing out $CdCl_2$ with aqueous ethanol, is tedious and difficult to reproduce because of emulsification. Furthermore, the product normally contains traces of lysolecithin, a hemolytic agent that can influence reactivity in screening tests which use erythrocytes. In other methods (2-4), the CLC procedure is circumvented by using column chromatography of crude egg yolk extract. These methods require high capacity columns and rechromatography of the product to remove lysolecithin and sphingomyelin.

This paper describes a modification of Pangborn's method that uses CLC to concentrate the phosphate containing lipids in the crude extract. The critical transformation of the CLC to lecithin and the removal of remaining impurities is done chromatographically in one step.

EXPERIMENTAL

Materials

Alumina (aluminium oxide 90, basic, Brockmann Grade I, 70-230 mesh ASTM) and materials for thin layer chromatography (TLC) analysis (precoated Silica Gel 60) were obtained from Merck. All chromatographic solvents used were of analytical purity (Merck).

Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and their methyl esters were obtained from Fluka. Palmitoleic acid (16:1), arachidonic acid (20:4) and their methyl esters were from Sigma. All the fatty acids and esters were of analytical purity.

Chromatographic Methods

Adsorption chromatography. Alumina (100 g) activated at 100 C overnight was packed in chloroform in a glass tube 40 cm long and 3.0 cm id. After packing (bed ht 12 cm), the bed was stabilized by elution with chloroform (150 ml). Chromatographic solvent was evaporated with a rotary vacuum evaporator under N_2 at 300 mm Hg and 30 C; these conditions provided good condensation of the toxic solvent vapors at the temperature of tap water (10 C).

Thin layer chromatography. TLC was performed using 5 µl samples, and chloroform/methanol/12.5 % aqueous ammonia (65:25:4 v/v) as solvent. After development, the plates were dried and the spots visualized with molybdophosphoric acid spray (Merck).

Gas liquid chromatography. GLC was performed with a Hewlett-Packard model 5840 A dual column gas chromatograph equipped with terminal 5840 A for electronic measurement of retention times and peak areas. The chromatograph was equipped with 2 glass

columns (1.8 x 2 mm id) filled with 10% polydiethylene glycol succinate on Chromosorb W AW 80/100. The columns were run with nitrogen (60 ml/min) and at temperatures programmed from 120 C to 190 C with a 4 C/min gradient. Injection temperature was 225 C and flame ionization detection was at 275 C.

Chemical Analysis

Phosphate determination was done according to Martin and Doty (5) using perchloric-acid-hydrolyzed samples. Nitrogen was estimated by the Kjeldahl method. Cadmium was estimated by EDTA-titration using Eriochromschwarz T as indicator (6). Dry weight determinations were made as described by Pangborn (7).

Fatty Acid (FA) Analysis

Solid lecithin was refluxed 2 hr with 2 N KOH in 50% aqueous methanol, acidified with 6 N HCl, extracted 3 times with petroleum ether (30-50 C) and washed 3 times with distilled water. Half of the petroleum ether extract was evaporated with N_2, dissolved in ethanol and the fatty acid (FA) was titrated as described by Hanahan (8). The other half was taken to dryness with nitrogen and esterified by refluxing the residue 45 min with 20% boron trifluoride in methanol (3 ml); distilled water (3 ml) was added and the mixture was extracted with 3 portions of 3 ml hexane. This extract was used for the GLC analysis. For preparation of standard fatty acid methyl ester (FAME), pure FA were weighed and esterified together as described for the lecithin FA. The FA for esterification were selected according to the normal FA content in egg lecithin and preliminary GLC analysis.

Purification of Lecithin

Yolks from 5 fresh hen eggs were stirred for a few seconds in a Waring blender. The yolks were extracted 3 times using 96% ethanol (190 ml) each time. The mixture was filtered in a Büchner funnel after each extraction. The clear, yellow, combined filtrate was set aside for 1 hr at 0 C in order to sediment impurities and was then filtered. To this primary extract was slowly added 50% aqueous $CdCl_2$ until no further precipitation occurred (ca. 6 ml). The mixture was centrifuged after 1 hr at room temperature. The precipitate was washed twice with 96% ethanol, dissolved in water-saturated diethyl ether (50 ml) and reprecipitated with 99.9% ethanol (50 ml) and centrifuged. This treatment was then repeated. The purified CLC was dissolved in chloroform (80 ml) and applied to the Alumina column; just before the sample level had fallen below the top of the bed, the sides of the column tube were washed with dichloromethane containing 7% methanol (v/v). Two portions of 10 ml each were used. This washing is essential to prevent precipitation of the CLC with the eluting solvent. After this washing, the eluting solvent (925 ml dichloromethane + 70 ml methanol + 5 ml 25% aqueous ammonia) was applied. The column was eluted with 950 ml of this solvent at a flow rate of 8 ml/min; the solvent was then switched to 100% methanol. A total of 13 fractions (100 ml each) were collected. Two preparations were made: D consisted of fractions 2-6; C consisted of fractions 7-11. The solvent was evaporated and the dry material dissolved in 99.9% ethanol, giving clear, colorless solutions.

RESULTS AND DISCUSSION

Five normal egg yolks contain ca. 30 g of lipid (9). As shown in Table I, extraction with 3 changes of ethanol takes out ca. one-third of this amount. A fourth extraction takes out more lipid, but only small amounts of lecithin are extracted as estimated from TLC analysis.

The dry weight determination (Table I) and the TLC analysis (Fig. 1a) show that almost all the lecithin is eluted in 500 ml (fractions 2-6). Figure 1a further shows that most of the impurities are retained by the column and are first eluted with the 100% methanol (fraction 12). Preparation D was chromatographically pure lecithin whereas preparation C contained traces of impurities (Fig. 1b); preparation C was therefore discarded. Preparation D was filtered through a sintered glass filter (porosity G-5) and stored in sealed ampoules. Table II shows the results of the chemical examination. Percent phosphorus (%P), percent nitrogen (% N), P/N mole ratio and FA/P mole ratio are within the theoretically calcd values for pure lecithin. No cadmium could be detected either in the FA extract or in the aqueous phase derived from

TABLE I

Dry Weight Determination on Fractions in Lecithin Purification

Fraction	Total Volume	Total Dry wt
	(ml)	(g)
A: Primary extract	566	9.8
B: Purified $CdCl_2$-lecithin complex	80	5.4
C: Fractions 7-11	6	0.3
D: Fractions 2-6	55	2.8

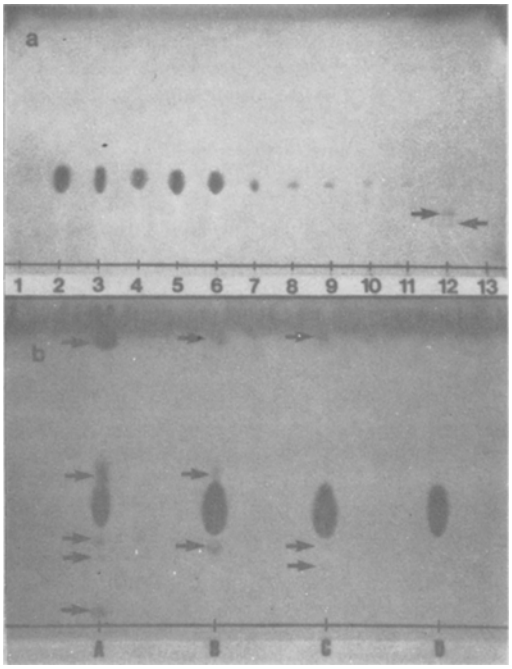

FIG. 1. TLC chromatograms showing (a) elution pattern of lecithin and impurities (arrows) from the Alumina column, and (b) that **A** (the primary extract), **B** (the purified $CdCl_2$-lecithin complex) and **C** (pool of fractions 7-11) contained decreasing amounts of impurities (arrows), whereas **D** (pool of fractions 2-6) was pure.

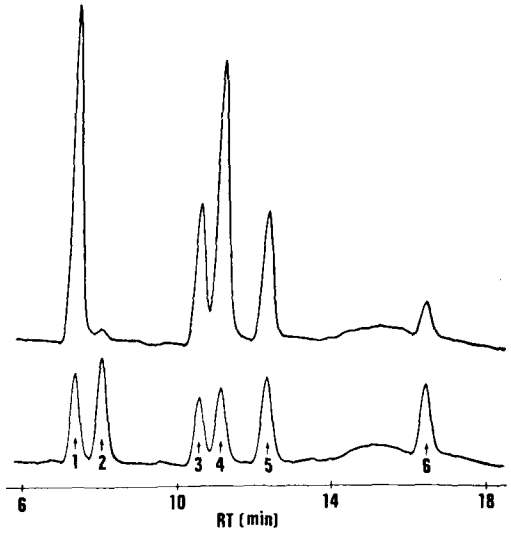

FIG. 2. GLC chromatogram of standard FAME (fatty acid methyl esters, lower curve) and lecithin FAME (upper curve). peak numbers are: 1, methyl palmitate; 2, methyl palmitoleate; 3, methyl stearate; 4, methyl oleate; 5, methyl linoleate and 6, methyl arachidonate.

lecithin hydrolysate. This agrees with information that more than 99% of the $CdCl_2$ applied to the Alumina column as the $CdCl_2$-lecithin complex was eluted with 6 N hydrochloric acid after methanol elution.

Figure 2 presents the GLC chromatogram obtained with standard FAME and lecithin FAME. The methyl esters in the standard FAME were also run separately and individual peaks were identified by their times of retention. The differences in retention times between individual peaks in standard FAME and the appropriate peaks in lecithin FAME were less than 0.1%, except for palmitoleic acid, where the difference was 0.5%. The 6 methyl esters in standard FAME gave different peak areas, (Table III) although the same amounts of the individual FA were taken for esterification. Therefore, response factors (relative to palmitic acid) for the individual FA were calculated and peak areas of the individual esters in lecithin FAME were corrected by multiplication with the appropriate relative response factor. The relative FA composition (wt %) in lecithin was then determined assuming the corrected peak area was proportional to the FA composition. The FA composition thus determined in the purified lecithin agrees with other reports (Table IV). The mean molecular weight (MW) for lecithin, based on the calcd FA composition was 772, which

TABLE II

Chemical Analysis on Preparation D (Pure Lecithin)

Fraction	This study	Theoretical
P %	3.99	4.01[a]
N %	1.79	1.81[a]
P/N-mol ratio	1.01	1.00
FA/P-mol ratio	1.98	2.00

[a]Based on mean molecular weight 772 g/mol for lecithin, see Results and Discussion.

TABLE III

Peak Area Obtained in GLC Experiment (Figure 2)

Peak No.	Standard FAME		Lecithin FAME peak area
	FA/ (w/w)	Peak area	
1	15.5	174,500	643,400
2	15.5	222,200	57,270
3	17.6	143,100	297,700
4	18.1	185,800	671,400
5	16.6	223,400	394,400
6	16.7	339,300	236,000

TABLE IV

Reported Fatty Acid Composition in Egg Lecithin

Fatty acid	This study	Ansell et al. (11)	Hasegawa and Suzuki (12)[a]
Palmitic	28.0	32	32.4
Palmitoleic	2.0	0	0.3
Stearic	17.9	16	16.6
Oleic	32.0	30	33.0
Linoleic	14.4	17	13.5
Arachidonic	5.7	4	4.0

[a]Values calcd from reported FA contents in monoacetyl-diglyceride from ovolecithin.

agrees with the % P and % N found (Table II).

The major peaks in the purified lecithin as shown in Figure 2 are compared in Table IV. The broad peak from lecithin FAME and standard FAME, located between peaks 5 and 6, was seen only in some of the runs. The inconsistent appearance of this peak, which had an area of 12% of the total area, is not understood. Minor peaks also were detected, but not identified. Altogher, 6 minor peaks were detected in the lecithin FAME. Total area of these minor peaks was 5% of the total area of all peaks detected. Neglecting the broad peak and the minor peaks, the identified fatty acids account for 95% of the FA in the purified lecithin.

This method for purification of lecithin gives chemically and chromatographically pure lecithin. The yield from 5 eggs was 2.8 g. This is the same amount obtained by Pangborn (1) (7 g from 12 yolks) and Rathlev (10) (0.4-0.5 g/yolk). This method can be scaled up using a larger column, is easy to reproduce and purification can be done in 1 day. Five separate runs of purification gave identical patterns in the TLC analysis (Fig. 1). The chemical data and GLC data presented in this paper are from 1 of the 5 runs.

The chromatographic method described would probably also be useful for purification of other lipids containing the phosphocholine moiety and for purification of synthetic lecithins.

ACKNOWLEDGMENT

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REVIEW

Lipid Oxidation: Biologic Effects and Antioxidants—A Review[1]

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ABSTRACT

The detection and measurement of lipid oxidation in biological systems and some biologic effects of this oxidation are reviewed. The role of lipid oxidation in the process of photocarcinogenesis and the protective effect of antioxidants against this process also are discussed. The mechanism of such protection is unknown and studies directed at elucidating the mechanism of antioxidant effect in photocarcinogenesis and in some other pathological conditions believed to involve lipid oxidation are needed. In addition to this, epoxidation of lipids observed in monolayer studies requires further investigation, particularly in the presence of some other unsaturated molecules. The possible significance of such a study—particularly in the presence of polycyclic aromatic hydrocarbon carcinogens, where formation of epoxides is generally accepted as active intermediates—is also discussed. In addition, present knowledge on the role of lipid peroxides in the destruction of proteins and biomembranes, in chemically induced toxicity and in generation of singlet oxygen is presented.

INTRODUCTION

It has long been known that fats go rancid by slow autoxidation during storage. In recent years, there has been a renewed interest in studying the mechanism of lipid oxidation and in the detection of oxidized products, particularly in biological systems. It is now recognized that lipid oxidation in biological membranes is a very destructive process. To date, lipid oxidation has been implicated in liver cell injury caused by chemicals (1-4) such as CCl_4, $BrCCl_3$, 1,1,2,2-tetrachloroethane, ethylene bromide and ethanol. Lipid peroxidation has been proposed as a possible mechanism in the clinically important phenomenon of ozone toxicity (5,6) in which lung damage induced by ozone and nitrogen dioxide results (7). In addition to these effects, reactions between peroxidized lipids and proteins have been shown to cause loss of enzyme activities (8,9), polymerization (10-14), polypeptide chain scission (15), accelerated formation of brown pigments (8,14,16) and the destruction of labile amino acid residues such as histidine, lysine, cysteine and methionine (12). Photosensitized oxidation of lipids has been invoked in the process of photocarcinogenesis (17-23). Black and Chan have reported that cholesterol-α-oxide is generated both in vitro and in vivo by irradiation of cholesterol in the presence of oxygen. Cholesterol-α-oxide is reported to show weak carcinogenic activity and was suggested as a proximate carcinogen in the process of photocarcinogenesis by these workers. In support of this postulate, Lo and Black (17,23) have reported that feeding a diet rich in antioxidants affords considerable protection against photocarcinogenesis and delays the growth of tumors in comparison to control animals fed unsupplemented diet.

In this article, lipid oxidation is reviewed in general with particular emphasis on some of its biologic effects. The role of antioxidants as protective agents against photocarcinogenesis and some other pathological conditions involving lipid oxidation also are discussed.

LIPID OXIDATION

Oxidation in Bulk Phase

"Dark" oxidation. Several studies on autoxidation of fatty acids have been reported previously (24-28). A mechanism which is now generally accepted is that autoxidation of lipids involves a free radical mechanism as shown in Figure 1. The oxidation is initiated by allylic $H^{\bullet}$ abstraction followed by oxygen attack on the carbon radical thus generated. In recent years, using gas chromatography (GC)-mass spectroscopy (MS), Frankel et al. (29-32) and others (33-35) have done a detailed

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$$RH + O_2 \longrightarrow R\cdot + {}^{\cdot}OH$$
$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$$
$$ROO\cdot + RH \longrightarrow ROOH + R\cdot$$
$$R\cdot + R\cdot \longrightarrow RR$$
$$R\cdot + ROO\cdot \longrightarrow ROOR$$
$$ROO^{\cdot} + ROO\cdot \longrightarrow ROOR + O_2$$

FIG. 1. Autoxidation of lipids: RH represents a fat molecule in which H is an allylic hydrogen.

study of the composition of autoxidation products of methyl oleate, methyl linoleate and methyl linolenate (Table I) and of their mixtures in different proportions. As shown in Table I, the major products obtained in autoxidation of methyl oleate include 8-, 11-, 9- and 10-hydroperoxides. It is interesting that concentrations of 8- and 11-hydroperoxides in these studies are higher than 9- and 10-hydroperoxides. Methyl linoleate, on the other hand, gives equal amounts of 9- and 13-hydroperoxides, indicating that initial $H^{\cdot}$ abstraction occurs at doubly allylic carbon 11. Bulk phase oxidation of methyl linolenate yields the expected products derived from abstraction of hydrogen radical from 9- and 11- carbons which are doubly allylic positions. However, the product distribution (9- and 16-OOH>>12- and 13-OOH) of hydroperoxides is not as expected by the general mechanism shown in Figure 1. On the basis of mechanistic studies reported up to this time, it appears that primary processes involved in autoxidation still conform to the general free radical mechanism shown in Figure 1; the final distribution of the products, however, would depend on secondary reactions such as rearrangement of the intermediate allylic radicals or of the final products, further oxidation and disproportionation reactions. In view of this, it has been suggested (32) that the reduced yield of 12- and 13-hydroperoxides (18-25%), in comparison to 9- and 16-hydroperoxides (75-81%) from methyl linolenate, may result from the unique 1,5-diene structure of the former group leading to the formation of the 6-membered cyclic peroxides or more likely by their tendency to cyclize into prostaglandin-like endoperoxides (Fig. 2). From the practical aspect, it implies that despite any secondary reactions involved, the bulk phase "dark" oxidation of lipids can be inhibited by free radical quenchers. The antioxidants commonly used in the food industry are 3(2)-*tert*-butyl-4-hydroxyanisole (BHA); 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT); 4-hydroxy-methyl-2,6-di-*tert*-butylphenol (Ionox-100); mono-*tert*-butylhydroquinone (TBHQ), 3,3′-thiodipropionic acid (TDPA); 2,4,5-trihydroxybutyrophenone (THBP); dilauryl thiodipropionate (DLTDP); *n*-propyl gallate (PG); and nordihydroguaiaretic acid (NDGA).

Photosensitized Oxidation

Although most fats and lipids do not absorb visible or near untraviolet (UV) light, photosensitized oxidation caused by chromophore

TABLE I

Autoxidation of Fatty Acids

Fatty acid	Reaction conditions	Principal products	Ref. no.
1. Methyl oleate	bulk phase; "dark" 25-80 C	Hydroperoxides, 8,11 > 9,10	30
2. Methyl oleate	bulk phase; photosensitized (erythrosine); room temp.	9 and 10 OOH	39
3. Methyl oleate	bulk phase; photosensitized (riboflavine); room temp.	8,9,10 and 11 OOH	39
4. Methyl linoleate	bulk phase; "dark"; 40-50 C	9 and 13 OOH (1:1)	31
5. Methyl linolenate	bulk phase; "dark"; 25-50 C	9 and 16 OOH (75-81%) 12 and 13 OOH (13-25%)	32
6. Methyl linolenate	bulk phase; photosensitized (erythrosine), room temp.	9, 12, 13 and 16 conjugated diene OH	39
7. Methyl linolenate	bulk phase; photosensitized (riboflavine), room temp.	9, 12, 13 and 16 conjugated diene OOH	39
8. Linoleic acid	monolayer on silica gel, 60 C	*cis*-9,10- and *cis*-12,13-monoepoxy linoleic acids	51
9. Linoelaidic acid	monolayer on silica gel, 60 C	*trans*-9,10- and *trans*-12,13-monoepoxy linoelaidic acid	51

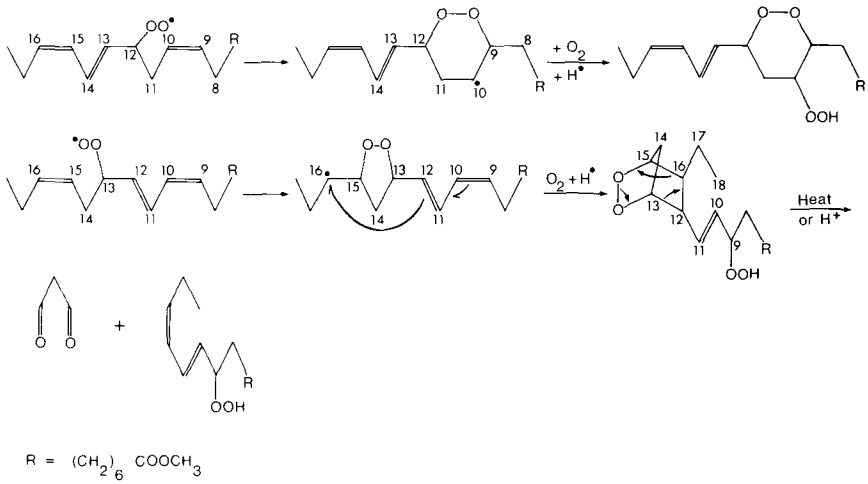


FIG. 2. Autoxidation of methyl linolenate: rearrangement of 12- and 13- hydroperoxides (32,69).

impurities present such as chlorophyll, porphyrins, myoglobins and pheophytins has long been known (36,37). In general, photosensitized oxidation of lipids is believed to involve singlet oxygen (37) produced by photosensitized excitation of triplet oxygen by chromophore impurities. Several mechanisms (38) such as "ene," radical-, ionic-, peroxirane- or dioxetane-intermediates have been proposed for addition of singlet oxygen to olefins. Of all the mechanisms proposed, the "ene" mechanism (Fig. 3) and peroxirane intermediates are most consistent with experimental facts (38). Chan (39) has reported, however, that some photosensitized oxidation of lipids may involve triplet state oxygen. Using 2 different sensitizers, erythrosine and riboflavine, for oxidation of methyl oleate and methyl linolenate, he showed that erythrosine sensitization involves singlet oxygen (Fig. 4, type II) whereas riboflavine-sensitized oxidations involve triplet oxygen (Fig. 4, type I). Since singlet oxygen reactions with nonconjugated olefins are known to involve 1,2-attack (Fig. 3) whereas oxidation by triplet oxygen involves free radicals, a distinction between 2 mechanisms was achieved by structural elucidation of the hydroperoxides formed, by the aid of GC-MS (39). Methyl oleate, upon photosensitized oxidation with erythrosine, afforded 9- and 10-hydroperoxides only whereas riboflavine-sensitized reactions gave a mixture of 8-, 9-, 10- and 11-hydroperoxides. Similarly, methyl linolenate generated a mixture of 9-, 12-, 13- and 16-conjugated diene hydroperoxides in both erythrosine- and riboflavine-sensitized reactions, except that erythrosine-sensitized reactions also afforded 10- and 15-nonconjugated diene hydroperoxides which were not observed at all in the riboflavine reaction. Since formation of nonconjugated diene hydroperoxides is possible only through singlet oxygen (attack at 10 or 15 position; Fig. 5), the erythrosine-sensitized reactions were considered to involve $^1O_2$. Riboflavine-sensitized oxidations primarily involve radical formation like "dark" oxidation but notable differences in the 2 processes were observed (39). Unlike "dark" oxidation, photooxidation involving triplet oxygen did not involve chain reactions. Besides, no induction period was observed in the photooxidations whereas the "dark" oxidations involve long induction period. This was supported by the relatively small inhibitory action of the antioxidant BHT in the riboflavine reactions. From the practical viewpoint, this implies that prevention of photosensitized oxidation involving type I or type II mechanisms should not be possible through the antioxidants commonly used to inhibit "dark" oxidation. Singlet oxygen quenchers such as carotene, triethylamine and nickel chelates are quite effective inhibitors of photooxidative deteri-

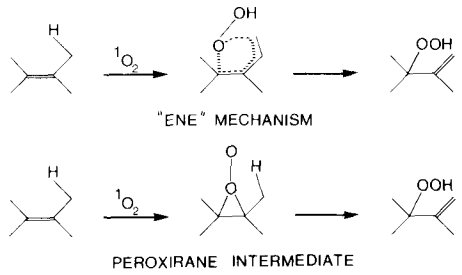


FIG. 3. Reaction of $^1O_2$ with olefins (38).

Sens. + A + hν → [intermediates I] Type I

[intermediates I] + O_2 → Products + Sens.

Sens. + O_2 + hν → [intermediates II] Type II

[intermediates II] + A → products + Sens.

Sens. + hν → ^{1}sens.

^{1}sens. → ^{3}sens.

^{3}sens. + $^3O_2$ → Sens. + $^1O_2$

FIG. 4. Proposed mechanism for photosensitized oxidation by triplet (Type I) and singlet (Type II) oxygen (39).

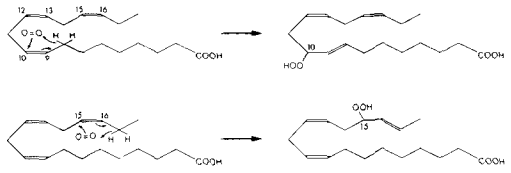


FIG. 5. Photosensitized (erythrosine) oxidation of linolenic acid. Formation of 10- and 15-nonconjugated diene hydroperoxides (39).

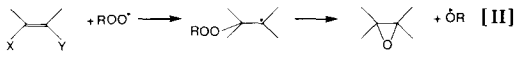
oration in lipids. Naturally occurring α-tocopherols quench singlet oxygen efficiently but are themselves oxidized in the process (40). Effective inhibitors for type I oxidation are not very well known.

Autoxidation in Monolayers

The use of adsorbed monomolecular films as models for the study of nonenzymatic membrane lipid autoxidation has been attempted by several investigators (41-51). Honn and coworkers (41) using silica gel as the support for soybean oil, were the first to correlate the effect of different ratios of substrate-to-solid support on autoxidation. Porter et al. (44,45) demonstrated that the maximal rate of autoxidation was exhibited by the linoleic acid-to-silica ratio close to that for a monolayer. Porter et al. (44,45) also studied the effects of prooxidants and antioxidants on the rate of lipid autoxidation.

Unlike oxidation in bulk phase, oxidation of linoleic and linoelaidic esters in monolayers form predominantly *cis* or *trans* epoxy compounds (51). Methyl oleate, however, does not give any detectable amount of the epoxy compound. Kinetically, the reaction is reported to be of first order in contrast to bulk phase oxidation where the reaction is kinetically more complex. Mechanistically, the reaction is rationalized as an addition of peroxy radical generated by initial abstraction of allylic hydrogen (Eq. I) on double bond. This is followed by loss of alkoxy radical from the intermediate, resulting in the formation of epoxides (Eq. II).

ROOH → ROO· + H· [I]



[II]

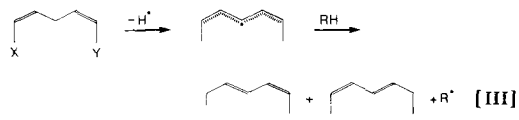
Since no detectable amount of the epoxide is observed in the monolayer oxidation of methyl oleate, it would be interesting to observe if methyl oleate epoxide is formed when a mixture of the esters of oleic and linoleic acids is oxidized. If interepoxidation reactions of this kind are observed in fatty acid esters, it will be of further interest to investigate their reactions with other molecules such as polycyclic aromatic hydrocarbons (52) and cholesterol (17-23), the epoxides of which have been implicated as "proximate" carcinogens in chemical and photocarcinogenesis, respectively. This kind of study may further shed light on the mechanism involved in enhancement of carcinogenic activity in the polycyclic aromatic hydrocarbons by unsaturated fatty acids (53).

DETECTION AND MEASUREMENT OF LIPID OXIDATION

Recently Gray (54) has reviewed the detection and measurement of lipid oxidation in vitro, specifically in food products. We shall therefore confine ourselves to biological systems only. Assays used in these systems can basically be divided into the following categories: (a) conjugated diene assay; (b) estimation of hydrocarbon gases; (c) detection of malonaldehyde and fluorescent products; and (d) loss of polyunsaturated fatty acids.

Conjugated Diene Assay

It has been observed by several workers that lipids containing dienes or polyenes on peroxidation show a shift in double bond position leading to conjugation (55-57). Mechanistically, it involves initial abstraction of H· from the doubly allylic position followed by double bond migration resulting in conjugated dienes which show an intense absorption at 233 nm (Eq. III). Similarly, conjugated trienes show an



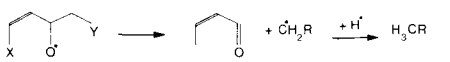
[III]

absorption at 288 nm. This has been widely used for detection and estimation of lipid peroxidation in liver cell injury by hepatotoxic agents. A limitation of the method is, however, that it is nonspecific and the extinction coefficients used for biological systems are only approximate.

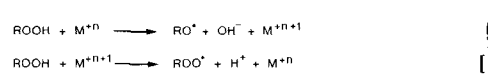
Other methods based on estimation of conjugated dienes use classical Diels Alder reactions. Ellis and Jones (58) used this method for estimation of conjugated dienes in tung oil. Maleic anhydride was used as a dienophile and quantitation was done by estimating the unreacted anhydride. This method, however, requires high temperatures and long reaction times, and is thus not suitable for biological systems. Waller and Recknagel (59) have successfully extended the scope of this reaction to biological systems by using ^{14}C-labeled tetracyanoethylene (TCNE) as an extremely reactive dienophile. Quantitative determination of conjugated dienes is done by estimating the incorporation of radioactive labels in the adducts. Limitations of the method are that: (a) conjugated trienes and tetraenes also form adducts with the dienophile, whereas *cis-cis* dienes do not make an adduct because of their transoid configuration; (b) phosphate groups react with TCNE (this difficulty was overcome, however, by reducing phosphate groups with lithium aluminium hydride before analysis); and (c) preparation of labeled TCNE involves labeled KCN, which is a deadly poison and requires extremely careful handling.

Hydrocarbon Gases

Riley et al. (60) first reported in *Science* in 1974 that hydrocarbon gases of low molecular weight (MW) were released upon treatment of mice with CCl_4. Since then, several reports have appeared indicating measurement of these gases as an index of lipid peroxidation in biological systems (61-66). A general route for the formation of these gases as visualized by Evans et al. (67) is shown in Equation IV. Hydroperoxide decomposition to alkoxy radical is the key step in the proposed scheme which is followed by β-scission and hydrogen abstraction resulting in the formation of hydrocarbon gases. β-Scission of alkoxy radical is a well known process (68) and involves unpairing of electrons in the bond located *beta* to the free radical. This process generates hydrocarbon free radicals and stable carbonyl compounds. As a support for this mechanism, it has been observed that transition metals, particularly the iron and copper catalysts, help form these gases in relatively greater amounts. Since free radicals of hydrocarbons can follow other routes for their termination besides hydrogen abstraction, a variety of products are theoretically possible. Most important of these alternative routes include dimerization, unsaturation caused by loss of $H^{\bullet}$ radical and further β-scission followed by the processes just mentioned. In view of this, it would be difficult to develop a quantitative relationship between state of peroxidation and the amount of a particular gas generated. Besides, calculated molar ratios show that hydrocarbon gases are only minor lipid oxidation products (69). Since $^{\bullet}OH$ is more stable as ^{-}OH whereas $H^{\bullet}$ preferably stays as H^{+}, it is conceivable that transition metals in their lower oxidation states would aid the formation of alkoxy radicals (Eq. V) whereas their higher oxidation states would favor generation of peroxy radicals (Eq. VI). Since, in biological systems, transition metals are present mostly in higher oxidation states, routes leading to hydrocarbon gases are of only minor importance. Despite these limitations, Tappel et al. and other workers (64-67) have devised methods for quantitative measurement of hydrocarbon gases and have indicated their use in estimating the extent of oxidation of lipids.



[IV]



[V]

[VI]

Detection of Malonaldehyde and Fluorescent Products

Detection of malonaldehyde (Fig. 6), commonly known as the thiobarbituric acid (TBA) test, has been used widely both in vivo (68) and in vitro (54) for the detection of lipid peroxidation. Experimental procedure involves treatment of oxidized lipid with thiobarbituric acid which results in the formation of a highly colored complex that is measured by colorimetric method (λ max 532 nm). Limitations

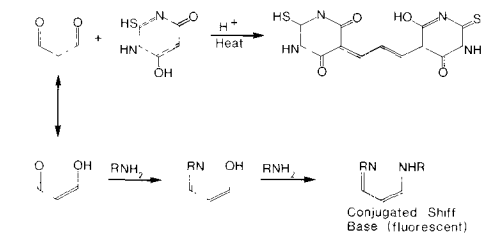


FIG. 6. Reaction of malonaldehyde with thiobarbituric acid and with compounds containing $-NH_2$ groups (69,74).

and pitfalls of this method in vitro are adequately covered by Gray (54) and may well hold for biological systems. Since malonaldehyde itself is a very reactive material and is known to make cross linkages with proteins in biological systems, it is questionable whether malonaldehyde is the ultimate product in lipid oxidation which reacts with TBA or if some other reactive material generates malonaldehyde under the conditions of TBA test. Pryor et al. (69) have suggested that, at least in part, the prostaglandin type of endoperoxides are a possible precursor of malonaldehyde under the test conditions. Since reaction of malonaldehyde with proteins and other cellular constituents results in fluorescent products, the detection of lipid oxidation in biological tissues by fluorescence has been found to be 10-to-100 times more sensitive than the TBA test (70-74). Although this method is very sensitive and is gaining wide acceptance, the chemistry of the formation of fluorescent products and their specific characterization needs further exploration.

Loss of Polyunsaturated Fatty Acids

This technique, developed by May and McCay (75), uses the loss of polyunsaturated fatty acids moieties as an index for the detection and measurement of lipid peroxidation. In this method, total fatty acid composition of the tissue lipid is determined by gas liquid chromatography (GLC) before and after lipid oxidation. The loss in amount of polyunsaturated fatty acids in oxidized lipid is then compared to that in the control. A major difference between this and other methods is that it involves a direct analysis of the tissue lipids themselves as opposed to the detection of products resulting from peroxidation. In this respect, this technique provides one of the most direct methods for detection and measuring the extent of lipid peroxidation in biological systems.

BIOLOGIC EFFECTS OF LIPID PEROXIDATION

Lipid Oxidation and Photocarcinogenesis

It is now widely accepted that UV light, specifically between 280-320 nm, can cause cancer (76). The mechanism of photocarcinogenesis is, however, not well understood. The basic mechanistic theories for photocarcinogenesis, DNA damage and repair, lysosomal destruction and photochemical mechanisms have been discussed in a recent review by Black and Chan (19). Although all these theories have their merits and demerits, we shall focus here on some aspects of photochemical theory which invokes sterol and lipid oxidation as the primary steps in the process of photocarcinogenesis. An intensive series of studies by Black and associates has centered around the observation that irradiation of skin, both in vitro and in vivo, leads to the photooxidation of sterols (77,78). One of the observed photoproducts, cholesterol-α-oxide, has been shown to be weakly carcinogenic (79), leading to the speculation that in vivo photooxidation might be the route to a "proximate carcinogen" of photocarcinogenesis. The mechanism for the formation of cholesterol-α-oxide is unknown and deserves further investigation as it may reveal the presence of some other active species involved in irradiation of skin. It has been suggested that the epoxide formation possibly involves free radicals because it was observed that cholesterol-5α,6α-epoxide levels in skin of animals fed an antioxidant- (free radical quencher) supplemented diet reached a peak 4 weeks after that of animals fed regular diet. Since formation of the epoxide is observed both in vitro (photolysis in water) and in vivo, its formation possibly involves an attack of peroxy radicals generated from cholesterol itself or from other lipids on the 5-6 double bond of cholesterol (Fig. 7). It may be recalled here that stereospecific formation of epoxy compounds is also observed in monolayer autoxidation of fatty acid methyl esters. Whatever the mechanism involved for the formation of the cholesterol epoxide, the subsequent studies of Black and Chan indicate there appears to be no direct relationship between light-induced formation of the epoxide and photocarcinogenesis. Moreover, the most effective wavelengths for the formation of the epoxide are reported to be 254 and 265 nm, which do not contribute to the solar spectrum reaching earth. Photosensitized reactions, however, involving triplet oxygen, as observed in riboflavine-sensitized oxidation of lipids (39), are possible in skin and deserve further exami-

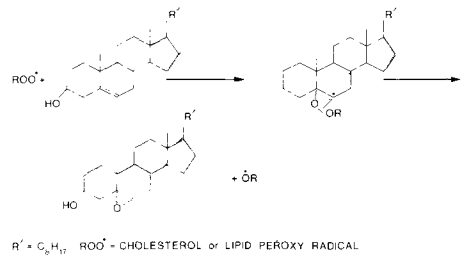


FIG. 7. Possible mechanism for the formation of cholesterol-5α,6α-epoxide.

nation. Without considering in detail the studies done by Black et al. and the associated interpretations, there are 3 specific observations in Black's work which invite further examination: (a) irradiation of normal skin with carcinogenic UV light led to oxidation of at least one normal constituent (78); (b) feeding a diet containing a mixture of added antioxidants delayed the appearance of photooxidation products (23); and (c) animals fed the antioxidant-containing diet were afforded considerable protection against photocarcinogenesis (17).

Whether or not a specific chemical mediator for carcinogenesis has been identified, the mechanism by which antioxidants could afford such protection is of interest. One possibility, certainly, is through direct or indirect screening of the skin. Since oral grooming is typical behavior in mice, and since the antioxidant mixture used made up 2% of the diet, detectable skin surface contamination is probable; alternatively, one or more of the components could have reached the skin indirectly through intestinal absorption and cutaneous excretion. Of the antioxidants used (BHT, ascorbate, reduced glutathione and α-tocopherol acetate), only the tocopherol (E max. 288 nm) is a likely candidate for chromophore status in normal light. DeRios et al. (80) have reported reduced erythema responsiveness in antioxidant-fed animals, but the light source in this case was a medium pressure mercury arc. Since shortwave (254 nm) UV light is a major contributor to the erythema effectiveness of this source, all components of the mixture could provide some protective screening.

That photochemical alteration of skin sterols has been observed in vitro (22) is evidence that such changes are not secondary effects of metabolic alterations. On the other hand, Black et al. (21) observed changes in metabolic activity of light-induced tumors compared to unirradiated skin, reflected in ^{14}C-acetate incorporation into various lipid classes. Whether the changes were tumor-specific or true of irradiated skin generally was not examined in this study. The possibility remains that any observed changes in cutaneous lipids of irradiated animals could involve direct or indirect photochemical effects or altered metabolic activity.

Most normal skin lipids are unlikely to function as chromophores for mid-UV light (> 290 nm); exceptions include carotenoids, some dehydrosterols and possibly other polyenes. Lipid peroxides were reported, however, by Dubouloz and Dumas (81) following irradiation of skin and others have reported enhanced free radical signals in irradiated skin (82). Thus, the likelihood of endogenously photosensitized reaction in skin does exist. Since oxidation of cholesterol and accumulation of its photoproducts reaches a maximum well before tumor appearance, it would be most reasonable to look for systematic cumulative lipid changes during early stages of irradiation.

Interaction of Lipid Peroxides with Proteins

The observation that malonaldehyde, an ultimate product in oxidation of polyunsaturated fatty acids, can make cross linkages with proteins (8,14,16,70) aroused a new interest in the study of lipid peroxides-protein interactions (8-16). Conversely, free radicals generated by peroxidation of lipids have been reported to initiate free radical formation in proteins which may, in turn, result in dimerization or polymerization (10-14). The polymerization process is considered to be more damaging to biomembranes. Most of the studies on lipid peroxide-protein interaction are done in vitro on sulfur amino acids because of the oxidative sensitivity of the sulfhydryl group. Roubal and Tappel (12) have reported that peroxidation can cause destruction, in varying degrees, of individual amino acids including arginine, serine, glutamic acid, methionine, tyrosine, phenylalanine and threonine. In studying the chemical nature of such interactions, Gardner et al. (83) has reported that N-acetyl cysteine, catalyzed by 10^{-5}M ionic iron in 80% ethanol, adds to linoleic hydroperoxide forming a thio bond. Reaction of a specific isomer of the hydroperoxide, 13-hydroperoxy-*trans*-11,*cis*-9-octadecadienoic acid, and N-acetylcysteine forms a number of products, 2 of which were identified as 9-S-(N-acetylcysteine)-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid (I) and 9-S-(N-acetylcysteine)-10,13-dihydroxy-*trans*-11-octadecenoic acid (II) (Fig. 8). Yong and Karel (84) have reported that reaction of histidine with methyl linoleate

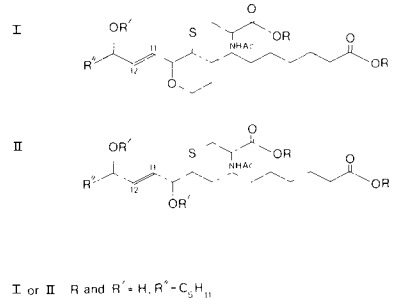


FIG. 8. Principal products of reaction between 13-hydroperoxy-*trans*-11, *cis*-9-octadecadienoic acid and N-acetylcysteine (81).

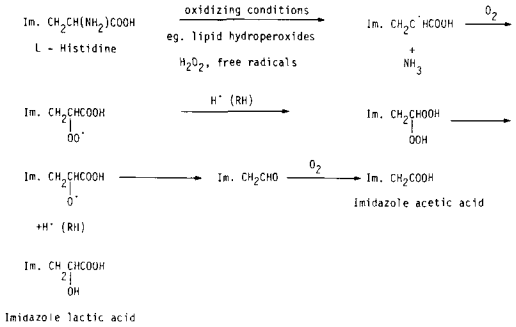


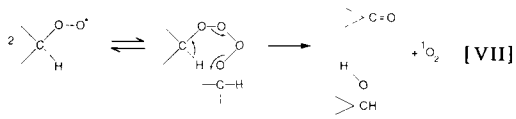
FIG. 9. Reaction of L-histidine and peroxidized lipids (82).

hydroperoxide (dispersed on a filter paper) affords imidazole lactic acid and imidazole acetic acid as major products. The reaction is considered to be taking place through free radical-mediated deamination and decarboxylation (Fig. 9). Nielson (85) has shown that interaction between peroxidized phospholipid (cardiolipin) and protein (albumin) results in covalent bonding.

The foregoing discussion clearly indicates that chemical interaction between peroxidized lipid and proteins is feasible but the nature of such interactions in vivo is not clear yet. More realistic model systems and further studies in vivo are required before any clear picture of these processes would emerge.

Generation of Singlet Oxygen

The finding by chemical means that singlet oxygen is generated in the self-reaction of *sec* butyl peroxy radicals (86) led to the speculation that peroxy radicals may be responsible for the generation of $^1O_2$ in the NADPH-dependent microsomal lipid peroxidation system (87). Nakano et al. have confirmed spectroscopically the generation of $^1O_2$ in such a system and have further demonstrated the generation of singlet oxygen from linoleic acid peroxy radicals. Peroxy radicals were produced from linoleic acid hydroperoxide by oxidation with ceric ion. The proposed mechanism (86,88) for the generation of singlet oxygen involves formation of tetroxide and seems to be in agreement with experimental observation (Eq. VII).



[VII]

Recently, it was shown that 2,5-diphenylfuran, when incubated with linolenic hydroperoxide in Tris buffer (pH 8.5) for 30 min, afforded *cis*-dibenzoylethylene (89), a product obtained by reaction of 2,5-diphenylfuran with singlet oxygen. It was inferred from these studies that a singlet oxygen-like factor was produced from linolenic acid hydroperoxide under the experimental conditions. Incubation of linolenic acid hydroperoxide, however, with diphenylanthracene (DPA) and 7,12-dimethylbenz[a]anthracene (DMBA) under the conditions described did not yield any detectable amount of endoperoxides (Logani, Austin, and Davies, unpublished results) or their rearranged products (90-92). Since it has been reported that formation of *cis*-dibenzoylethylene from 2,5-diphenylfuran may not necessarily involve singlet oxygen (93), the use of more specific traps is required to establish the generation of singlet oxygen from lipid hydroperoxides. Although at this time the hazards of singlet oxygen in biological systems are not very well defined (94), the presence of this active specie can be quite damaging to biological membranes and has been implicated in photocarcinogenesis (95) and photodynamic action (94,96).

Chemically Induced Toxicity

It has long been suspected that toxicity caused by several chemicals involve lipid peroxidation (1-4). The major limitation in these studies has been to detect and quantitate the amount of peroxidation directly in vivo. Because of the instability of organic peroxides, direct quantitation of lipid hydroperoxides cannot be relied upon in biological systems. Different analytical methods for quantitation and detection of lipid peroxides and their limitations have been discussed previously in this article and a combination of several techniques is therefore recommeded to obtain any reliable results. Despite these limitations, there is growing evidence that lipid peroxidation is induced by several chemicals, particularly by hepatotoxic agents. Based on conjugated diene assay, it has been shown that liver injury induced by CCl_4,$BrCCl_3$, 1,1,2,2-tetrachloroethane, ethylene dibromide and ethanol involves lipid peroxidation (50). The increase in expired ethane levels was further used by Riley et al. (60) to demonstrate the involvement of lipid peroxidation in CCl_4 toxicity. Participation of peroxides in liver injury caused by ethanol has been indicated by Di Luzio and Hartman (97) using increased formation of malonaldehyde in liver homogenates of rats as an assay for lipid peroxidation. More recently, Litov et al. (3) have supported these results using the increased levels of pentane as an index of lipid peroxidation. A similar increase in

levels of hydrocarbon gases (ethane and pentane) has been reported when rats were exposed to 1 ppm level of ozone for 1 hr (3).

The effect of antioxidants in modifying the influence of these chemicals appears to support the peroxidation (free radical) theory. Di Luzio (98) demonstrated that pretreatment with antioxidants inhibited ethanol-induced fatty liver. A similar effect of vitamin E on production of ethane and pentane has been observed in ozone and ethanol toxicity (3). In further support of the peroxidation theory, N,N′-diphenyl-*p*-phenylenediamine (DPPD), an efficient free radical quencher, was found to significantly delay the effect of vitamin E deficiency in male Sprague-Dawley rats (66). Despite all this evidence in support of peroxidation hypothesis, the mechanism and the significance of peroxidation induced by chemicals is unknown and deserves further investigation.

CONCLUSIONS

The implication that lipid peroxidation is involved in the process of photo carcinogenesis in the destruction of proteins in biological membranes and enzymes and in the chemically induced toxicity is supported by detection of free radical signals of inhibiting the effect of antioxidants and above all by detection of products considered to be originating specifically from lipid peroxides only. The use of modern sophisticated analytical techniques such as electron spin resonance (ESR), nuclear magnetic resonance (NMR), carbon magnetic resonance (CMR), GC-MS, isotopic labeling, high pressure liquid chromatography (HPLC), UV and fluorescent spectroscopy has greatly aided in detection and characterization of peroxidation products. Except for the characterization of lipid oxidation products, the conclusions based on detection of free radicals or on the effect of antioxidants are by inference only. Moreover, most of the studies are done in vitro where the effects of other components present in biological systems have not been taken into account. For instance, Wu et al. (99) have shown that the rate of disappearance of unsaturated fatty acids in the autoxidation as monolayers on silica gel per se changes considerably when palmitic acid, cholesterol or cholesteryl palmitate is incorporated in the system. Similarly, DNA has been reported to retard the rate of lipid oxidation in microsomal suspensions (100). Furthermore, at this stage, the significance of peroxidation in terms of different pathological conditions is not apparent. Although the significance of peroxidation in pathology is inferred from the inhibitory effect of antioxidants, the mechanism of such inhibition is unknown in most of the cases. For example, there is evidence from chemical carcinogenesis studies that antioxidants can inhibit aromatoc hydrocarbon carcinogenesis. The antioxidants, BHA and thio compounds (disulfuranne, dimethyldithiocarbonate, benzyl thiocyanate), have been reported to inhibit mammary tumorigenesis by benzpyrene (101). BHA reportedly inhibits binding of benzpyrene to DNA whereas the related compound BHT stimulates the activity of mixed function oxidases reputedly involved in carcinogenic activation (101). Studies directed at elucidating the mechanism of antioxidant effect are therefore needed to unravel the significance of lipid oxidation in the etiology of photocarcinogenesis and in other biological effects discussed here.

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Factors Affecting Fatty Acid Oxidation in Bovine Mammary Tissue[1]

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ABSTRACT

Oxidation of fatty acids was studied in bovine mammary tissue slices in order to evaluate their potential contribution to energy metabolism. Rates of fatty acid oxidation decreased with increasing chain length: acetate > octanoate > palmitate or oleate. Rates of oxidation of long chain, but not short chain, fatty acids increased over time, which could not be explained by carnitine palmitoyltransferase (CPT) activity. This phenomenon is not an artifact of the incubation system or caused by substrate solubility, as rates of palmitate oxidation were constant in rat kidney cortex slices. Preincubating mammary tissue with or without unlabeled palmitate showed that increasing rates of palmitate oxidation is not caused by use of endogenous fatty acids. Palmitate at 0.26 mM, equivalent to arterial fatty acid concentration, gave maximal rates of oxidation. The β-oxidation enzymes may restrict fatty acid oxidation as oxidation of [1-^{14}C] palmitate exceeded that of [U-^{14}C] palmitate. Acetate inhibited palmitate oxidation (75%) but not esterification, suggesting that acetate inhibits palmitate oxidation by substrate competition at the mitochondrial level or via malonyl-CoA inhibition of CPT. Glucose inhibited palmitate oxidation (67%) and stimulated esterification. Low palmitoyl-CoA levels would favor glyceride synthesis over oxidation, since the apparent K_m for palmitoyl-CoA of the glycerol-3-phosphate acyltransferases is lower than that for CPT. Thus, glucose presumably diverts palmitate from oxidation to glycerolipids. Clofenapate, a glyceride synthesis inhibitor, decreased triacylglycerol formation, and marginally increased palmitate oxidation. We estimated that long chain fatty acids can potentially account for 6-10% of the oxidative metabolism of mammary tissue.

INTRODUCTION

Milk synthesis is a very energy-demanding process and during the peak of lactation dairy cows are often in negative energy balance. Milk fat represents a loss of energy to the cow. Therefore, by increasing the oxidative metabolism of fatty acids in mammary cells, more energy would be available for the production of more milk and milk protein. This would be especially useful to high-producing cows at a peak of lactation since production may be limited by the extent of energy deprivation. However, Annison et al. (1) could not detect oxidation of long chain fatty acid in the mammary gland of fed lactating goats, but long chain fatty acids comprised up to 40% of oxidative metabolism in mammary gland of fasted lactating goats (2).

Thus, in order to assess the feasibility of stimulating fatty acid oxidation in mammary gland, the primary objective of this study was to determine what factor(s) regulates fatty acid oxidation in bovine mammary tissue.

MATERIALS AND METHODS

Tissue Collection and Incubation

Tissue slices were incubated in Krebs-Ringer bicarbonate (KRB) buffer with 0.7 mM $CaCl_2$ (3). Buffers were made the night before use and adjusted to pH 7.4 prior to collection of tissue. The incubation buffer contained bovine serum albumin (BSA Fraction V, fatty acid-poor, Sigma Chemical Co., St. Louis, MO). It was determined that palmitate oxidation was unaffected by varying BSA concentration in the media. In consideration of the 4 strong fatty acid binding sites on BSA, it was decided to keep the fatty acid-to-BSA molar ratio constant at 4.

Palmitate and oleate were prepared for incubation as follows: (a) 1.0 ml of [1-^{14}C]-palmitate, [1-^{14}C] oleate, or [U-^{14}C] palmitate (free acids, Amersham Corp.) in benzene (.01 mCi) was dried under N_2; (b) palmitate or oleate (free acid, Sigma Chemical Co.) was added to the desired concentration; and (c) the mixture was dissolved in 1.0 ml of absolute ethanol and stored at 4 C prior to use. [1-^{14}C]-Octanoate (sodium salt, Amersham Corp.) was stored in ethanol, and to this solution, octanoate (sodium salt, Sigma Chem. Co.) was added to the desired concentration. [1-^{14}C]-Acetate (sodium salt, Amersham Corp.) plus acetate (sodium salt, Sigma Chem. Co.) was stored in distilled water at -15 C prior to use. A 0.01-ml aliquot of the fatty acid in ethanol solution was added to 3 ml of KRB buffer in 25-ml flasks in a shaking water bath (37 C, 60 cycles/min, Dubnoff Metabolic Shaker). The flasks were then gassed for 10-15 sec with

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O_2/CO_2 (95:5) and capped with rubber stoppers fitted with plastic center wells (Kontes Glass Co.) containing a 2-cm^2 fluted filter paper. The flasks were then left shaking to allow dissolution of fatty acid. Rates of palmitate oxidation were equivalent when ethanol was used as a carrier as opposed to the aqueous ammonia salt of palmitate (4).

Mammary tissue pieces were excised from udders of lactating Holstein cows, obtained from the university herd or a local abbatoir. Cows were usually fed until time of slaughter, but they may have been fasted 1 day in some cases. A 3-day fast reduced palmitate oxidation to about one-half that in tissue from fed cows (data not shown). The tissue pieces were immediately placed in an insulated flask containing 37 C KRB buffer (2 units oxytocin/100 ml, Sigma Chem. Co.). Tissue was then rinsed in 37 C buffer (no oxytocin), cubed, trimmed to minimize nonparenchymal tissue, and sliced using a Stadie-Riggs microtome to yield slices of ca. 0.5 mm in thickness. The slices were placed in a common flask containing 37 C buffer, then rinsed several times. Slices were gently blotted to remove excess fluid, trimmed to minimize nonparenchymal tissue, weighed (usually 60 to 80 mg), then added to incubation flasks to commence incubation. Treatments within experiments were run in quadruplicate flasks and values were averaged.

Production of $^{14}CO_2$ was corrected against blanks (flasks incubated for 0 min with tissue). Incubation was terminated by injecting 0.3 ml of 5 N H_2SO_4 into the media followed by injection of 0.3 ml of methyl benzathonium hydroxide (Hyamine Hydroxide®, Sigma Chem. Co.) into the center well to trap CO_2. The flasks were shaken for 1 additional hr, after which the center well and its contents were transferred to scintillation vials. Samples were counted in a Nuclear-Chicago Model 720 liquid scintillation counter for 2 10-min counts in 10 ml of Aqueous Counting Scintillant (ACS, Amersham Corp.). Counting efficiency, using [^{14}C] benzoic acid (New England Nuclear) as an internal standard, was 82.5% in the presence of 0.3 ml of Hyamine Hydroxide.

Lipid Extraction and Chromatography

After CO_2 was collected, tissue slices from 4 flasks were pooled and rinsed 4 times in distilled water to remove "loosely attached" fatty acids. Lipids were then extracted from the tissue slices with chloroform/methanol (2:1) according to Folch et al. (5). A 0.5-ml aliquot of the chloroform/methanol extract was counted in 10 ml of ACS. Counting efficiency was 75%. Recovery of [1-^{14}C] palmitate by the Folch method was 95%. Total recovery of dpm as CO_2, tissue and media was 93-98% of that added. The lipid extract was washed twice with 0.2 vol of 0.05 M KCl, and a 0.5-ml aliquot counted in 10 ml of ACS. Counting efficiency was 79%. Accumulation of radioactivity in the aqueous phase was less than 10% of the total activity in the tissue. Excess anhydrous Na_2SO_4 was added to the chloroform phase to remove "trapped" water. The chloroform solution was then dried under N_2 and resuspended in 0.3-0.5 ml of chloroform/methanol (2:1) for thin layer chromatography (TLC).

A 30-50 μl aliquot of the lipid extract was spotted on Silica Gel-60 TLC plates (0.25 mm gel thickness, precoated on glass, E.M. Merck Co.) to determine esterified fatty acid fractions. The TLC plates were developed with hexane/diethyl ether/glacial acetic acid (70:30:2). A neutral lipid standard (Sigma Chem. Co.) containing monoglyceride, 1,2- and 1,3-diglycerides, triglyceride and fatty acid was cochromatographed. The plates were then dried and sprayed with 0.2% dichloroflourescein in ethanol for visualization. The spots were scraped off and counted in 10 ml of ACS to determine percentage distribution. The efficiency of elution of radioactivity from the gel was essentially 100% and quenching was nil.

Carnitine Palmitoyltransferase (CPT, EC 2.3.1.21)

CPT activity was assayed spectrophotometrically in mammary mitochondria according to Bieber et al. (6). Mammary mitochondria from 3 cows were assayed for CPT with varying concentrations of carnitine and palmitoyl-CoA (Sigma Chem. Co.) in order to determine appropriate substrate concentrations. Enzyme activity was linear with protein concentrations to 0.24 mg/ml. Protein was determined by the Lowry et al. method (7). L-Carnitine was a generous gift to Dr. L.L. Bieber of the Biochemistry Department of Michigan State University.

Statistical Analysis

Experiments were blocked according to treatment and time. Statistical evaluations were by analyses of variance, orthogonal contrasts and t-tests. For some sets of data, there was mild heterogeneity of variance even after log transformation. Standard statistical tests, however, are known to be relatively valid in spite of departures from the assumption of homogeneous variance.

RESULTS AND DISCUSSION

When investigating what controls a meta-

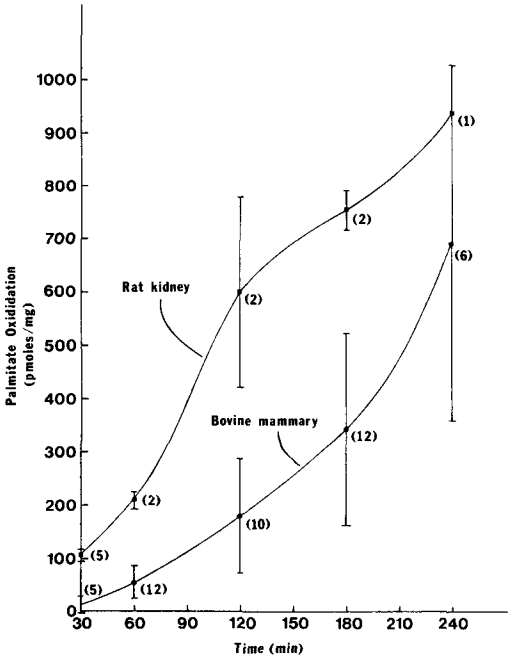

FIG. 1. Palmitate oxidized to CO_2 (pmol substrate/mg tissue) as a function of time for bovine mammary and rat kidney tissue. Vertical bars indicate standard error.

bolic pathway in vitro, it is necessary to establish maximal reaction conditions for the incubation system to be used. Palmitate oxidation per mg tissue was unaffected by amount of tissue, BSA and ethanol in the incubation media within limits used, and only slightly enhanced by gassing the media prior to incubation (data not presented). However, the rates of palmitate oxidation in mammary tissue slices were found to increase in a curvilinear fashion over time,

TABLE I

Effect of Glucose and Acetate on Palmitate Oxidation and Esterification

Treatments	CO_2[a]	GL[a]
	(pmol palmitate/mg x min)	
Palmitate	1,2[b,d]	9.7[d]
P + Glucose	0.4[e]	12.3[e]
P + Acetate	0.3[e]	9.6[d]
P + G + A[c]	0.3[e]	12.3[e]

[a]Standard errors of difference between means for CO_2 and GL (glycerolipid, mono-, di-, triglyceride + phospholipid) are 0.10 and 0.74 and number of replicates is 6).

[b]Rates are average of those obtained at 60, 120 and 180 min although oxidation typically doubled between 60 and 180 min.

[c]Palmitate, glucose and acetate at 0.26, 2.8 and 0.6 mM.

[d,e]Means in columns with different superscripts are different ($P < 0.01$).

shown in Figure 1. Bovine data were obtained from animals that were most likely fasted for 1 day prior to slaughter. However, the rates of palmitate oxidation are similar to those obtained from fed animals (8) (Tables I and II).

Barac-Nieto (9), using similar incubation conditions, found that rat kidney cortex slices oxidize 0.98 mM palmitate at a constant rate of 8.3 pmol/mg x min. Thus, in order to determine if this increasing oxidation rate with time was unique to mammary tissue or an artifact of the incubation system, the rate of palmitate oxidation was determined in rat kidney cortex slices in the same manner as that determined for mammary tissue slices. The data in Figure 1 show that rates of palmitate oxidation in rat kidney cortex slices were nearly constant over time. Thus, increasing rates of palmitate

TABLE II

Effect of Clofenapate and Glucose on Palmitate Oxidation and Esterification

Treatments	CO_2[a]	DG[a]	TG[a]	GL[a]
	(pmol palmitate/mg x min)			
Palmitate	0.5[e]	1.2	6.4[g]	8.7
P + glucose	0.2[f]	3.0	6.9[g]	11.9
P + clofenapate[b]	0.7[e]	1.8	3.5[h]	7.0
P + G + C[c]	0.3[f]	3.3	1.7[h]	7.2

[a]Standard errors of difference between means for CO_2, DG (diacylglycerol), TG (triacylglycerol), and GL are 0.07, 0.53, 0.86, 1.39 and number of replicates is 5 for CO_2 and 3 for other parameters. Incubation was 60 min.

[b]Clofenapate was a generous gift of Imperial Chemical Industries, Ltd., of London, England.

[c]Palmitate, glucose and clofenapate at 0.26, 2.8 and 0.5 mM.

[e,f,g,h]Means in columns with different superscripts are different ($P < 0.10$).

oxidation with increasing time of incubation is not an artifact of the incubation conditions.

Since mammary tissue fatty acid concentrations of 4 mM have been reported (10), use of endogenous fatty acids with time of incubation could account for increasing rates of palmitate oxidation. To test this hypothesis, mammary tissue slices were preincubated for various times without substrate, after which palmitate was added. The rate of ^{14}C-palmitate oxidation doubled between 60 and 180 min in the tissue slices preincubated for 0 min (Table III). However, preincubation of tissue slices for 60 min maximally stimulated palmitate oxidation and abolished the effect of time on palmitate oxidation consistent with removal of competition from endogenous substrate. The presence of palmitate during preincubation should block palmitate oxidation by replenishing endogenous pools of fatty acid. Mammary tissue slices were again preincubated for various times, but unlabeled palmitate was present during preincubation after which [1-^{14}C]-palmitate was added. Rate of oxidation tripled between 60 and 180 min with 0 min preincubation (Table IV). However, preincubation of the tissue slices for 60 min with unlabeled substrate maximally stimulated palmitate oxidation and abolished the effect of incubation time. Therefore, increasing rates of palmitate oxidation with time are independent of the presence of substrate prior to incubation.

The effect of substrate concentration on palmitate oxidation in mammary tissue slices is shown in Table V. Rates of palmitate oxidation were maximal at 0.26 mM at 60 and 180 min. This level of palmitate would correspond to arterial plasma fatty acid concentrations in the fed cow (8). Also the rate of palmitate oxidation doubled between 60 and 180 min at all concentrations tested. Half-maximal velocity was reached at ca. 0.1 mM. Therefore, maximal rates of palmitate oxidation are obtained with physiological fatty acid concentrations and the effect of incubation time is independent of substrate concentration expected in mammary tissue. Thus, it seems that in mammary tissue fatty acid oxidation is not regulated by the fatty acid concentration normally present.

A comparison was made of the effect of fatty acid chain length on fatty acid oxidation (Table VI). The data clearly show that oxidation decreases as chain length increases up to C16. Acetate is oxidized at substantially greater rates than is palmitate, oleate or octanoate, which agrees with Bickerstaffe et al. (8) who found that acetate is the preferred oxidative substrate in the bovine mammary gland. Swenson and Dimick (11) found substantial rates of medium chain fatty acid oxidation in the goat mammary gland. Annison et al. (1) could not detect oxidation of long chain fatty acids

TABLE III

Effect of Preincubating Mammary Tissue Slices in Buffer with No Substrate on Palmitate Oxidation[a]

Preincubation (min)	Time with substrate (min) 60 (pmol substrate/mg x min)	180 (pmol substrate/mg x min)
0	0.7[b,d]	1.4[b,e]
30	1.0[c]	1.9[c,e]
60	1.5[d]	1.7
90	1.1	1.4

[a]Standard error of difference between means is 0.21, and number of replicates is 3.

[b]At 0 min pre-incubation $0.7 < 1.4$ ($P < 0.01$).

[c]At 30 min pre-incubation $1.0 < 1.9$ ($P < 0.01$).

[d]At 60 min with substrate $1.5 > 0.7$ ($P < 0.01$).

[e]At 180 min with substrate $1.9 > 1.4$ ($P < 0.05$).

TABLE IV

Effect of Preincubating Mammary Tissue Slices with Unlabeled Palmitate on Palmitate Oxidation[a]

Pre-incubation (min)	Time (min) 60 (pmol substrate/mg x min)	180 (pmol substrate/mg x min)
0	0.6[b,c]	1.8[b]
30	1.4	2.0
60	2.1[c]	2.0
90	2.2	2.0

[a]Standard error of difference between means is 0.39 and number of replicates is 2.

[b]At 0 min preincubation $0.6 < 1.8$ ($P < 0.05$).

[c]At 60 min $2.1 > 0.6$ ($P < 0.05$).

TABLE V

Palmitate Oxidation vs Palmitate Concentration

Concentration (mM)	Time (min) 60	180
0.065	0.3[a]	0.6[b]
0.13	0.5[x]	1.2[c,y]
0.26	0.8[x]	1.8[d,y]
0.33	0.8[x]	1.8[d,y]

[a]Standard error of difference between means is 0.26 and number of replicates is 4.

[b,c,d]Means in columns with different superscripts are different ($P < 0.10$).

[x,y]Means in rows with different superscripts are different ($P < 0.02$).

(LCFA) in the mammary glands of fed goats. Palmitate and oleate are oxidized at equal rates. Oxidation of octanoate and acetate changes marginally with time, whereas oxidation of palmitate and oleate is doubled by incubation time.

Differences in rates of oxidation with different chain length can be explained by several reasons. Short chain and medium chain fatty acids can be activated in the intramitochondrial-matrix space, thus bypassing the carnitine-mediated transport mechanism, whereas LCFA oxidation is carnitine-dependent, since they are activated on the outside of the inner mitochondrial membrane (12). This suggests that carnitine palmitoyltransferase (CPT) activity limits fatty acid oxidation. Also, LCFA, by virtue of their site of activation, are accessible to the competing esterification pathway. The higher rates of octanoate oxidation and/or the lack of increasing oxidation rate with time may indicate regulation of LCFA at the level of β-oxidation since the results of others suggest that LCFA oxidation is regulated by the β-oxidation enzymes (13-15). In summation, short and medium chain fatty acids are oxidized at much greater rates than LCFA, and increasing oxidation with time is unique to LCFA.

In order to determine whether rates of palmitate oxidation were limited by CPT activity, tissue slices were incubated with palmitate for various times after which mitochondria were isolated and assayed for CPT activity according to Bieber et al. (6). The results of Table VII show that CPT activity is not influenced by incubation time, although this does not prove that the effective activity in vitro was altered. However, it can be estimated that the CPT activities are much greater than maximal rates of palmitate oxidation. Therefore, limitation of palmitate oxidation by CPT activity seems unlikely, which is consistent with studies on isolated liver mitochondrial preparations (16-19).

If LCFA oxidation is limiated by the β-oxidation enzymes, then greater rates of oxidation with [1-^{14}C] palmitate in comparison to [U-^{14}C] palmitate would be expected because of accumulation of partially degraded acids. The data in Table VIII indeed reveal that oxidation of [1-^{14}C] is greater than [U-^{14}C]-palmitate. Also, time of incubation appears to have a more pronounced effect on [U-^{14}C]-palmitate oxidation, nearly a 3-fold increase. Moreover, Waterson and Hill (15) showed that enoyl-CoA hydratase demonstrated much greater substrate specificity for short and medium chain substrates than for long chain substrates. Thus, it appears that the enzymes of β-oxidation may limit palmitate oxidation.

Acyl-CoA esters are at a branchpoint be-

TABLE VI

Fatty Acid Oxidation vs Chain Length

Fatty acid[a]	Time (min) 60 (pmol substrate/mg x min)	180 (pmol substrate/mg x min)
Palmitate	0.7[b,d]	1.3[d]
Oleate	0.7[d]	1.3[d]
Octanoate	9.1[e]	11.3[e]
Acetate	49.7[c]	57.7[c]

[a]Palmitate, oleate and octanoate at 0.26 mM, acetate at 0.6 mM.

[b]Standard error of difference between means is 0.56 and number of replicates is 4 for palmitate, oleate and octanoate. Rats are not on a gram-atom carbon basis.

[c]Rates are average of 3 separate experiments from animal sources which differ from those for palmitate, oleate and octanoate; standard deviations are 17.9 and 17.8 for 60 and 180 min.

[d,e]Means in columns with different superscripts are different ($P < 0.01$).

TABLE VII

Effect of Incubation Time on Carnitine Palmitoyltransferase (CPT) Activity

	Time (min) 0	60	180
CPT activity	10.15[a,b]	8.82	7.63

[a]Rates expressed as nmol CoA formed/mg mitochondrial protein x min.

[b]Standard error of difference between means is 3.17 and number of replicates is 5.

TABLE VIII

Oxidation of [1-^{14}C] Palmitate vs [U-^{14}C] Palmitate

Isotope	Time (min) 60 (pmol substrate/mg x min)	180 (pmol substrate/mg x min)
[1-^{14}C] palmitate	0.7[a,b,x]	1.2[b,y]
[U-^{14}C] palmitate	0.3[c,x]	0.8[c,y]

[a]Standard error of difference between mean is 0.11 and number of replicates is 4.

[b,c]Means in columns with different superscripts are different ($P < 0.01$).

[x,y]Means in rows with different superscripts are different ($P < 0.05$).

tween acylation to *sn*-glycerol-3-phosphate (G3P) or carnitine. Blood glucose serves as the source of G3P in mammary tissue (20) and the G3P pathway is the major pathway for the formation of triacylglycerols in milk (10,21,22). Acetate is quantitatively the most important source of metabolic energy in the ruminant mammary gland. Acetate may inhibit palmitate oxidation by competing for the available pool of free CoA in the mitochondria (23) or cytoplasm, or by contributing to malonyl-CoA accumulation, which inhibits CPT (24,25). Thus, it was of prime importance to determine the fate of exogenous palmitate in mammary tissue slices incubated in the presence of glucose and acetate.

As shown in Table I, palmitate oxidation was markedly inhibited by glucose and acetate. The decreased palmitate oxidation in the presence of glucose was associated with a concomitant increase in palmitate esterification. Rao and Abraham (26) also found similar changes in palmitate use with glucose in mammary tissue slices from lactating mice. As mentioned, palmitoyl-CoA is at a branchpoint between esterification to G3P or carnitine. The apparent K_m for palmitoyl-CoA for CPT in bovine mammary tissue is 21 μM as shown in Figure 2. However, Kinsella and Gross (27) found apparent K_m of 4 μM palmitoyl-CoA for the acyl-CoA:G3P acyltransferase enzyme. Thus, both enzymes exhibit very high affinities for palmitoyl-CoA, but that of G3P acyltransferase is higher. In turn, at relatively low palmitoyl-CoA concentrations, esterification would be the favored route, which is consistent with data in Table I in which rates of esterification are much greater than those of oxidation. Although acyl-CoA concentration was not directly determined in mammary tissue, the level of long chain acyl-CoAs at the mitochondrial surface would presumably be lower than fatty acid concentration in the media. Indeed, Tubbs and Garland (28) reported long chain acyl-CoA levels of 53 μM in rat liver. Also, the apparent K_m for G3P is 0.26 mM for G3P acyltransferase (27) and lactating bovine mammary tissue levels of 0.14 mM for G3P have been reported (29). So it would be expected that supplying precursors of G3P in the media would enhance fatty acid esterification. Levels of G3P doubled in mammary tissue slices from lactating mice when incubated with glucose (26). In summation, glucose seems to inhibit palmitate oxidation by diverting palmitate to glycerolipids.

The data in Table I show that acetate inhibits palmitate oxidation, but rates of palmitate esterification are unchanged. Thus, it

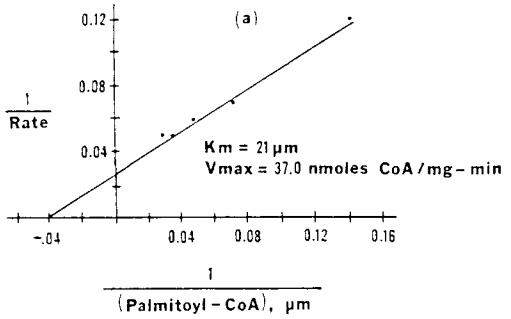

FIG. 2. Double reciprocal plot of substrate concentration vs carnitine palmitoyltransferase activity in mammary mitochondria.

appears that acetate blocks palmitate oxidation by competing with the mitochondrial pool of CoA or by inhibiting CPT via malonyl-CoA. Since acetate does not inhibit palmitate esterification, acetate probably does not compete with palmitate for the available pool of cytoplasmic CoA.

It has been shown that as rates of palmitate esterification increase, rates of palmitate oxidation decrease. Clofenapate, a derivative of clofibrate, at 0.5 mM reduced the incorporation of [^{3}H]glucerol into lipid fraction by 50% in liver slices (30). Therefore, the effect of blocking palmitate esterification by inclusion of 0.5 mM clofenapate into media on palmitate oxidation was investigated. As shown in Table II, clofenapate significantly inhibited triacylglycerol (TG) formation both in the presence and absence of glucose, whereas total glycerolipid (GL) formation was slightly depressed. However, rates of palmitate oxidation were only slightly enhanced by clofenapate. This could be the result of clofenapate selectively inhibiting diacylglycerol acyltransferase (30), which causes a build-up of partial glycerides (phospholipids, monoacylglycerols and diacylglycerols). Therefore, clofenapate was effective in reducing triacylglycerol formation, but tended to augment palmitate oxidation.

Using typical rates of palmitate oxidation at 180 min for mammary tissue slices obtained from cows in which total udder weight was determined, it was estimated that palmitate oxidation could potentially account for 15 ml of O_2 consumption by the whole udder per min using a RQ of 0.70 for the oxidation of lipids. This rate of O_2 consumption is 6.3% of the rate of O_2 uptake (243 ml/min) by the udder of fed lactating cows (8). Using the higher rates of palmitate oxidation, fatty acids could potentially account for 10% of the oxidative metabolism of mammary tissue. These estimates

would represent only an upper limit on fatty acid oxidation and would normally be much less because of inhibition by acetate and glucose.

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Glucagon and $N^6,O^{2'}$-Dibutyryl Adenosine 3':5'-Monophosphate Inhibition of Lipogenesis and Phosphofructokinase Activity of Hepatocytes from Meal-fed Rats

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ABSTRACT

Glucagon and $N^6,O^{2'}$-dibutyryl adenosine 3':5'-monophosphate (dibutyryl cyclic AMP) inhibit net glucose utilization, lactate plus pyruvate accumulation and fatty acid synthesis by isolated hepatocytes prepared from meal-fed rats. A crossover in the metabolite profile of the glycolytic intermediates occurs between fructose-6-phosphate and fructose-1,6-bisphosphate, suggesting either inhibition of phosphofructokinase or activation of fructose diphosphatase, or both. Direct assay of the enzymes in cell-free extracts of the hepatocytes indicates that dibutyryl cyclic AMP inhibits phosphofructokinase but has no effect upon fructose diphosphatase. The assay for phosphofructokinase was modified by the use of ITP in place of ATP for the phosphate donor as the ATP-linked assay is complicated by an apparent time-dependent activation of the enzyme. These findings strongly suggest that cyclic AMP inhibition of phosphofructokinase explains in part cyclic AMP inhibition of aerobic glycolysis and lipogenesis by rat liver hepatocytes.

INTRODUCTION

We have earlier suggested that an important feature of regulation of hepatic fatty acid synthesis is the level of lactate and pyruvate generated by glycolysis (1-3). Glucagon and $N^6,O^{2'}$-dibutyryl cyclic AMP (dibutyryl cyclic AMP) inhibit both aerobic glycolysis (1-6) and fatty acid synthesis by isolated hepatocytes (1-7). We have further proposed that cyclic AMP inhibition of glycolysis is achieved at the same enzymatic steps responsible for the stimulation of gluconeogenesis (1,2). Crossover analysis of the intermediates of glycolysis in the meal-fed rat liver (1) is identical to that of the gluconeogenic pathway from lactate of the fasted rat liver (8,9) and points to sites between fructose-6-phosphate and fructose-1,6-bisphosphate and between phosphoenolpyruvate and pyruvate. Inhibition of pyruvate kinase explains the latter crossover point, this enzyme being subject to inactivation by a cyclic AMP-dependent, phosphorylation mechanism (10-12). This enzyme has now been implicated both in the stimulation of gluconeogenesis (12) and in the inhibition of glycolysis and fatty acid synthesis (3). On the other hand, the mechanism responsible for the crossover between fructose-6-phosphate and fructose-1,6-bisphosphate elicited by cyclic AMP has not been clearly resolved. It could be explained by inhibition at phosphofructokinase (13,14), activation of fructose diphosphatase (15) or both (16). Inhibition of phosphofructokinase by a cyclic AMP-dependent mechanism appears to account in large part for the stimulation of gluconeogenesis by glucagon (17,18).

Previous studies from this laboratory failed to show any change in phosphofructokinase or fructose diphosphatase activity with hepatocytes incubated with cyclic AMP (2). However, this study reports that with proper conditions for extraction and assay of the enzyme, phosphofructokinase of isolated hepatocytes prepared from meal-fed rats is converted to a less active form by glucagon and dibutyryl cyclic AMP. In contrast, a change in fructose diphosphatase activity could still not be demonstrated. The apparent inactivation of phosphofructokinase by glucagon and dibutyryl cyclic AMP is suggested to account in part for the previously shown (1-6) inhibition of aerobic glycolysis and fatty acid synthesis by these compounds. While the study was in preparation for publication, Clark et al. (19) reported that dibutyryl cyclic AMP inhibition of glycolysis by hepatocytes prepared from chicken liver can be explained by inactivation of phosphofructokinase.

MATERIALS AND METHODS

Isolation of Hepatocytes, Incubation and Preparation of Digitonin Extracts

Hepatocytes were prepared from meal-fed female Wistar rats (240-280 g) by the Berry and Friend method (20) with modifications described previously (1). Incubations were carried out with 120-200 mg wet wt of hepatocytes in 25 ml Erlenmeyer flasks in a final vol of 4 ml of Krebs-Henseleit saline supplemented with 2.5% albumin (charcoal treated and dialyzed, Fraction V, Sigma Chemical Co., St. Louis, MO) at 37 C in an atmosphere of 95% O_2

and 5% CO_2. After 15 min of incubation, the hepatocytes were sedimented by centrifugation for 30 sec in a clinical centrifuge. The supernatant was discarded and the pellets suspended with a Potter-Elvehjem homogenizer with 1 ml of a solution 50 mM in morpholinopropane sulfonic acid, pH 7.0, 0.2 M in sucrose, 1 mM in EDTA and 3.25 mM in digitonin. The suspension was centrifuged 1.5 min in an Eppendorf centrifuge and the supernatant removed. This digitonin extract of the hepatocytes was stored on ice (up to 4 hr) before assaying phosphofructokinase and fructose diphosphatase activity.

Assay of Phosphofructokinase and Fructose Diphosphatase

A split beam spectrophotometer (Cary 118) was used. All components of the phosphofructokinase assay with the exception of fructose-6-phosphate were mixed with an aliquot of the digitonin extract of the hepatocyte. From this solution, 0.95 ml was withdrawn for both sample and reference cuvettes. A fructose-6-phosphate solution (50 µl) was then added to the sample cuvette and water (50 µl) to the reference cuvette. The difference in absorbance was recorded at 30 C as a function of time. This method of assay circumvented the rapid NADH oxidation displayed by digitonin extracts of hepatocytes. The final assay contained a suitable aliquot of the digitonin extract of the hepatocytes, 0.25 µmol of ATP or ITP, 5 µmol of $MgCl_2$, 0.15 µmol of NADH, 32 units of triose phosphate isomerase (E.C. 5.3.1.1), 1.5 units of glycerol phosphate dehydrogenase (E.C. 1.1.1.8), 0.41 units of aldolase (E.C. 4.1.2.7), 50 µmol of N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.4 and various concentrations of fructose-6-phosphate. Because digitonin extracts of hepatocytes contain all the glycolytic enzymes, the possibility was considered that fructose-1,6-bisphosphate produced by phosphofructokinase was converted in part to lactate rather than quantitatively to glycerol-3-phosphate. This was shown not to be a problem in an experiment in which the reaction was followed spectrophotometrically for NADH use and then stopped with perchloric acid. Lactate and glycerol-3-phosphate were measured by the Hohorst et al. method (21). Accumulation of lactate did not occur whereas the accumulation of glycerol-3-phosphate was that expected from the use of NADH.

For the assay of fructose diphosphatase, sample and reference cuvettes were set up as already described. The reaction was conducted at 30 C and initiated by the addition of fructose-1,6-bisphosphate and water to the sample and reference cuvettes, respectively. The final assay (1 ml) contained an aliquot of the digitonin extract of the hepatocytes, 50 µmol of TES, pH 7.4, 5 µmol of $MgCl_2$, 0.15 µmol of $NADP^+$, 10 units of phosphoglucose isomerase (E.C. 5.3.1.9) and 4 units of glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49).

For both assays, linearity with amount of extract used was established. The amounts of coupling enzymes were empirically determined to give maximal rates of reaction.

Assay of Metabolites

Solutions of fructose-1,6-bisphosphate were standardized enzymatically using the assay mixture described for phosphofructokinase. Solutions of fructose-6-phosphate were standardized in the same manner by adding purified rabbit muscle phosphofructokinase. Lactate and pyruvate were assayed by the Hohorst et al. method (21), glucose by the Slein method (22) using NAD^+ instead of $NADP^+$ and the NAD^+ linked glucose-6-phosphate dehydrogenase (Type XXI), glucose-6-phosphate and fructose-6-phosphate were assayed by the Lang and Michal method (23) and fructose-1,6-bisphosphate by the Michal and Beutler method (24).

Determination of Fatty Acid Synthesis

Rates of fatty acid synthesis were determined by the incorporation of tritiated water, as described previously (1).

Sources of Material

Chemicals were of the highest purity available. Enzymes from Sigma were partially desalted by centrifugation of the $(NH_4)_2SO_4$-saturated suspensions and reconstitution with water.

RESULTS

Inhibition of Aerobic Glycolysis by Dibutyryl Cyclic AMP

As shown in previous studies from this laboratory (1-3), dibutyryl cyclic AMP inhibits net glucose use by hepatocytes prepared from meal-fed rats (Table I). This effect correlates with a diminished accumulation of lactate plus pyruvate and is further characterized by an increase in glucose-6-phosphate plus fructose-6-phosphate and a decrease in fructose-1,6-bisphosphate levels (Table I). The decreased fructose-1,6-biphosphate results suggest that either phosphofructokinase is inhibited or fructose diphosphatase is activated as a consequence of elevated levels of cyclic AMP. Thus,

TABLE I

Effect of Incubation of Hepatocytes with Dibutyryl Cyclic AMP on Rate of Glucose Utilization[a]

Measurement	Results obtained: Without dibutyryl cyclic AMP	With 50 μM dibutyryl cyclic AMP
Net glucose utilization[b]	71 ± 5	7 ± 10*
Lactate accumulation[c]	1.14 ± 0.16	0.16 ± 0.09*
Pyruvate accumulation[c]	0.32 ± 0.04	0.05 ± 0.02*
Glucose-6-P plus fructose-6-P[d]	0.35 ± 0.02	0.66 ± 0.06*
Fructose-1,6-bisphosphate[d]	0.017 ± 0.001	0.011 ± 0.001*

[a]Incubations were conducted for 60 min except in the case of the phosphorylated intermediates which were determined after 30 min of incubation. Results are means ± SEM for 3 hepatocyte preparations from meal-fed rats. Statistical comparisons were made by the Student's t-test for paired data: *$P < 0.05$.

[b]Net glucose used calcd from the rate of glycogen used minus glucose released, μmol/hr/g wet wt.

[c]Concentration in the incubation medium after 60 min of incubation, μmol/ml.

[d]Concentration in the cells after 30 min of incubation, μmol/g wet wt.

the activities of these enzymes in cell-free extracts prepared from hepatocytes which had been treated with dibutyryl cyclic AMP were investigated.

Effect of Dibutyryl Cyclic AMP on the Fructose Diphosphatase Activity of Isolated Hepatocytes

Fructose diphosphatase activity, assayed with fructose-1,6-bisphosphate at an initial concentration of 25 μM, was not different for digitonin extracts prepared from hepatocytes incubated with or without 50 μM dibutyryl cyclic AMP (without 3.2 ± 0.6; with 3.0 ± 0.6 μmol/min/g wet wt; means ± SEM for 3 hepatocyte preparations). Furthermore, no difference in fructose diphosphatase activity resulting from incubation of hepatocytes with dibutyryl cyclic AMP could be demonstrated over a wide range of fructose-1,6-bisphosphate concentrations (Fig. 1).

Effect of Dibutyryl Cyclic AMP on the Phosphofructokinase Activity of Isolated Hepatocytes

Although negative results were obtained for any effect of dibutyryl cyclic AMP on fructose diphosphatase, evidence was found for dibutyryl cyclic AMP inactivation of phosphofructokinase (Table II and Figs, 2-5). Provided that low fructose-6-phosphate concentrations were used and 5′-AMP was not included in the assay medium, extracts of hepatocytes incubated with dibutyryl cyclic AMP consistently gave lower rates of phosphofructokinase activity. Representative absorbance changes corresponding to NADH use are given in Figure 2 for the phosphofructokinase assay conducted with and without 5′-AMP and with ATP as the phosphate donor. Without 5′-AMP, a striking difference was observed between the activities of digitonin extracts prepared from hepatocytes incubated without dibutyryl cyclic AMP (Fig. 2A) and hepatocytes incubated with dibutyryl cyclic AMP (Fig. 2B). Although dibutyryl cyclic AMP clearly had the effect of decreasing enzyme activity, it was difficult to express the results quantitatively because activity of the enzyme continually increased with time during the assay. Nevertheless, Figure 3 could be constructed from such data by plotting "initial

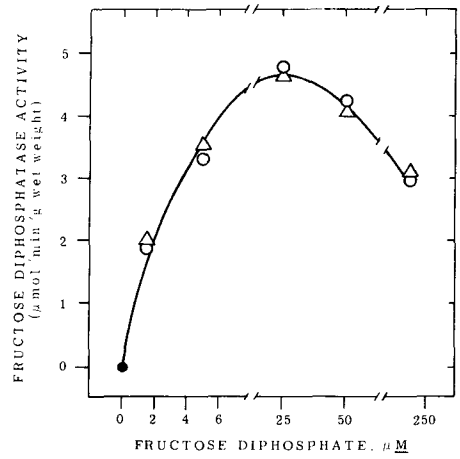

FIG. 1. Fructose diphosphatase activity vs fructose diphosphate concentration. Digitonin extracts of hepatocytes incubated without (○) and with (△) dibutyryl cyclic AMP for 15 min were used to assay enzyme activity.

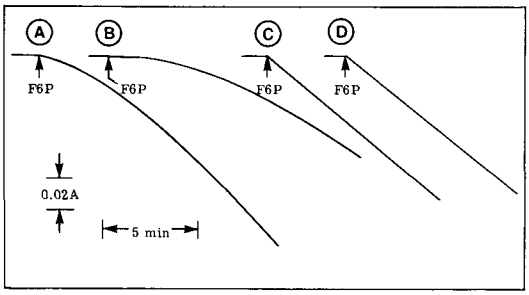

FIG. 2. Time course of phosphofructokinase assays with ATP as the phosphate donor. Traces show the changes in absorbance occurring with time in the phosphofructokinase assay. Digitonin extracts of hepatocytes incubated for 15 min with or without dibutyryl cyclic AMP (50 μM) were used. The initial fructose-6-phosphate concentration was 0.125 mM; 5′-AMP concentration was 1 mM, when used. A: hepatocytes incubated without dibutyryl cyclic AMP, phosphofructokinase assay conducted with 5′-AMP; B: hepatocytes incubated with dibutyryl cyclic AMP, assay conducted without 5′-AMP; C: hepatocytes incubated without dibutyryl cyclic AMP, assay conducted with 5′-AMP; D: hepatocytes incubated with dibutyryl cyclic AMP, assay conducted with 5′-AMP.

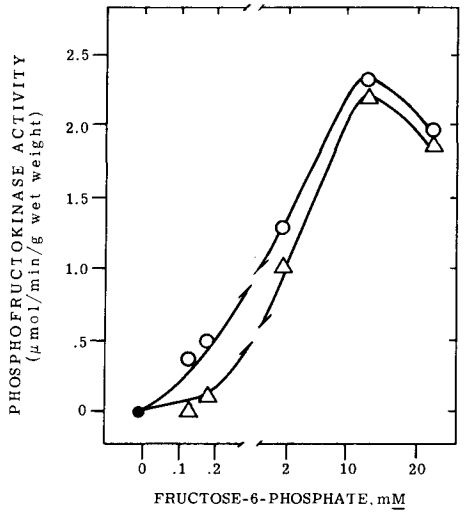

FIG. 3. Phosphofructokinase activity vs fructose-6-phosphate concentration with ATP as the phosphate donor. Digitonin extracts of hepatocytes incubated for 15 min without (○) and with (△) 50 μM dibutyryl cyclic AMP were used.

rates" obtained under these conditions; the initial rate is defined as the velocity for the first min after initiation of the reaction with fructose-6-phosphate. As shown in Figure 3, a substantial difference is apparent at low fructose-6-phosphate concentrations between the phosphofructokinase activity of control and dibutyryl cyclic AMP-treated hepatocytes. The difference becomes much less at higher fructose-6-phosphate concentrations, indicating that the major effect of exposure of cells to cyclic AMP is to shift the fructose-6-phosphate saturation curve for the enzyme to the right, having less effect upon the maximum velocity.

With 5′-AMP in the assay medium or fructose-6-phosphate at concentrations greater than 0.4 mM (not shown), phosphofructokinase activity does not increase with time (Figs. 2C and 2D). However, when assayed in the presence of 5′-AMP (Figs. 2C and 2D) or in the presence of high fructose-6-phosphate concentrations (Fig. 3), the activity of the enzyme was almost the same for digitonin extracts prepared from hepatocytes incubated with or without dibutyryl cyclic AMP. Thus, the greatest difference was noted under assay conditions where the enzyme undergoes a transition to a more active form. We found that the problem of apparent phosphofructokinase activation during the assay could be circumvented by using ITP in place of ATP as the phosphate donor. As shown in Figure 4, linear rates were obtained with ITP replacing ATP, with

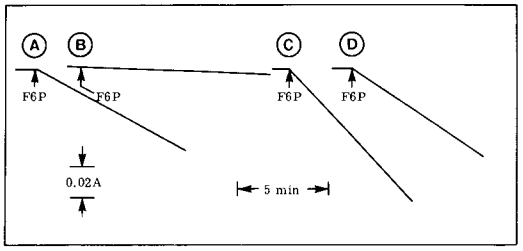

FIG. 4. Time course of the phosphofructokinase assay with ITP as the phosphate donor. Conditions were identical to those of Figure 2 (See legend to Fig. 2) except ITP was substituted for ATP in the assay for phosphofructokinase.

or without 5′-AMP in the assay. Digitonin extracts of hepatocytes incubated with dibutyryl cyclic AMP and assayed with ITP as phosphate donor also gave lower rates of phosphofructokinase activity than extracts of control hepatocytes (Fig. 4A and 4B). Including 5′-AMP in the assay greatly blunted the difference caused by the cyclic nucleotide (Figs. 4C and 4D). Table II summarizes the results obtained with 3 preparations of hepatocytes. The digitonin extracts were assayed for enzyme activity at a low initial concentration of fructose-6-phosphate (0.125 mM) with and without 1 mM 5′-AMP. A striking difference in phosphofructokinase activity between control and dibutyryl cyclic AMP-treated hepatocytes was observed when 5′-AMP

TABLE II

Effect of Incubation of Hepatocytes with Dibutyryl Cyclic AMP on the Activity of Phosphofructokinase[a]

Addition to the hepatocyte incubation	Phosphofructokinase activity	
	Without 5′-AMP	With 5′-AMP
	μmol/min/g wet wt	
None	0.21 ± 0.03	0.53 ± 0.03
Dibutyryl cyclic AMP	0.07 ± 0.01[b]	0.45 ± 0.02[c]

[a]Hepatocytes were incubated with dibutyryl cyclic AMP (50 μM) for 15 min. Digitonin extracts, prepared as described in Materials and Methods, were assayed for phosphofructokinase with ITP as the phosphate donor and 0.125 mM fructose-6-phosphate. The final concentration of 5′-AMP when included in the assay was 1 mM. Results are expressed as means ± SEM for 3 hepatocyte preparations and comparisons for significance made using the Student's paired t-test.

[b]$P < 0.05$, when compared to control (i.e., none) without 5′-AMP added to the phosphofructokinase assay.

[c]$P < 0.05$, when compared to control (i.e., none) with 5′-AMP added to the phosphofructokinase assay.

was excluded from the assay mixture. When 5′-AMP was present, a much smaller difference, albeit statistically significant by the Student's t-test for paired samples, was observed.

Although the velocity traces of phosphofructokinase activity were linear over time with ITP but not ATP as the phosphate donor, the resulting fructose-6-phosphate saturation curves obtained were qualitatively the same for both (compare Fig. 3 obtained with ATP and Fig. 5 obtained with ITP). Thus, regardless of the phosphate donor used to assay the enzyme, the major effect of incubation of hepatocytes with dibutyryl cyclic AMP is to shift the fructose-6-phosphate saturation curve to the right, only a very small difference being observed at saturating levels of fructose-6-phosphate. Hence, cyclic AMP modifies the enzyme such that more fructose-6-phosphate is required for a given level of activity until the enzyme is saturated with this substrate.

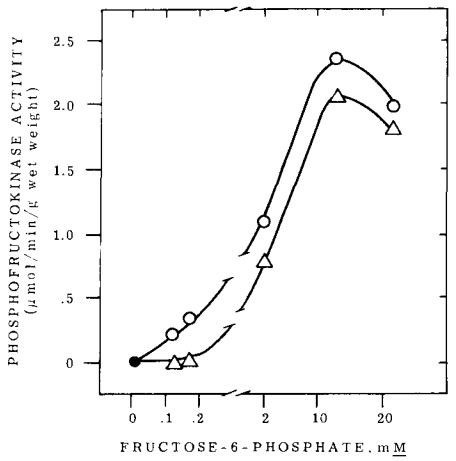

FIG. 5. Phosphofructokinase activity vs fructose-6-phosphate concentration with ITP as the phosphate donor. Digitonin extracts of hepatocytes incubated for 15 min without (○) and with (△) 50 μM dibutyryl cyclic AMP were used.

Inhibition of the Activity of Phosphofructokinase by Glucagon

The activity of phosphofructokinase of isolated hepatocytes was also found to be sensitive to glucagon. With 3 hepatocyte preparations incubated and assayed for phosphofructokinase activity as described in Table II, control hepatocytes gave values of 0.21 ± 0.03, whereas hepatocytes treated for 15 min with 10^{-6} M glucagon gave values of 0.08 ± 0.01 μmol/min/g wet wt ($P < 0.05$). As shown in Figure 6, half-maximal inhibition occurred at ca. 10^{-9} M glucagon under the conditions of these experiments. This glucagon titration curve is similar to those reported previously from this laboratory (2,3) for glucagon inhibition of net glucose use, lactate and pyruvate accumulation, pyruvate kinase activity and fatty acid synthesis.

DISCUSSION

This study demonstrates that the phosphofructokinase activity of isolated hepatocytes is decreased by dibutyryl cyclic AMP and glucagon. The activity of the opposing enzyme, fructose diphosphatase, does not appear to be affected. Since dibutyryl cycle AMP and glucagon cause a crossover in the metabolite

profile between fructose-6-phosphate and fructose-1,6-bisphosphate, inactivation of phosphofructokinase appears to explain in large part inhibition of aerobic glycolysis by these compounds. Inhibition of glycolysis also results in inhibition of lipogenesis. This is clearly shown by the fact that adding exogenous lactate plus pyruvate overcomes some of the inhibition of fatty acid synthesis imposed by dibutyryl cyclic AMP. However, it is not the entire story because much higher concentrations of exogenous lactate and pyruvate are necessary in the presence of dibutyryl cyclic AMP to restore a comparable rate of fatty acid synthesis. This means that another site of cyclic AMP inhibition has to be involved and recent findings (7,25,26) make it likely that the site is acetyl-CoA carboxylase.

Velocity traces obtained in the assay of phosphofructokinase with ATP as the phosphate donor were consistently nonlinear. Apparent activation of the enzyme occurred throughout the time course of the measurements. This held only below a fructose-6-phosphate concentration of 0.4 mM and was not observed in assays conducted with ITP as the phosphate donor. This may be because all of the adenine nucleotides are allosteric effectors of phosphofructokinase (27); ATP inhibiting at high concentrations, ADP activating, and 5′-AMP relieving ATP inhibiton. Because ITP does not function as a nucleotide regulator for the reaction catalyzed by phosphofructokinase but can serve as the phosphate donor (28), it was used as an alternative to ATP in many of the studies reported here.

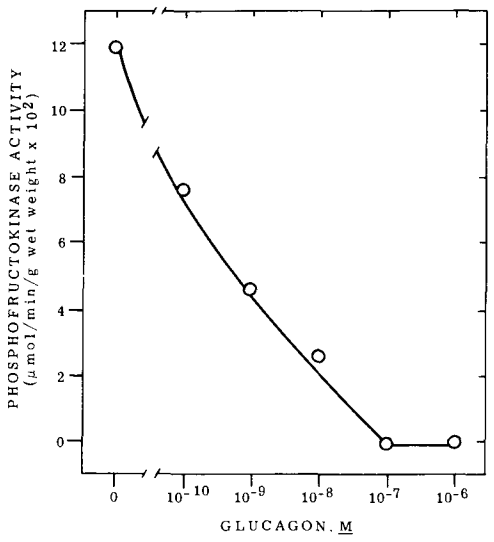

FIG. 6. Effect of glucagon in hepatocyte incubations on the activity of phosphofructokinase. Hepatocytes were incubated with various concentrations of glucagon for 15 min. Digitonin extracts were made as described in Materials and Methods and assayed for phosphofructokinase activity with ITP as the phosphate donor and fructose-6-phosphate at 0.125 mM.

Differential extraction of phosphofructokinase from the hepatocytes by digitonin might be argued as a possible explanation for the difference in activity between control and glucagon- or dibutyryl cyclic AMP-treated hepatocytes. Digitonin creates pores in the plasma membrane large enough for most enzymes to escape, but not large enough for the release of the polymeric form of acetyl-CoA carboxylase (E.C. 6.4.1.2) (29). However, this is not a factor with the release of phosphofructokinase because comparable differences in phosphofructokinase activity between control and dibutyryl cyclic AMP-treated hepatocytes were obtained with 1% Triton X-100 replacing digitonin in the extraction procedure. Furthermore, differential extraction should lead only to a change in V_{max} and not to a change in the concentration of fructose-6-phosphate required to half saturate the enzyme. Finally, lactate dehydrogenase was released by digitonin to the same extent with both control and dibutyryl cyclic AMP-treated hepatocytes, indicating that a change in the release of enzymes per se was not induced by dibutyryl cyclic AMP and glucagon.

Problems in the assay of phosphofructokinase of crude extracts accure in part from the necessity of using an indicator reaction coupled to the generation of fructose-1,6-bisphosphate. This compound is not only a substrate for fructose diphosphatase, but is also an activator of phosphofructokinase (30). Yet, direct assay of both enzymes indicates phosphofructokinase rather than fructose diphosphatase as the locus of cyclic AMP action. A large difference in phosphofructokinase activity between digitonin extracts prepared from control and dibutyryl cyclic AMP-incubated hepatocytes was observed without but not with 5′-AMP in the assay mixture. The reason for this effect of 5′-AMP is not completely understood. 5′-AMP is routinely included in the assay mixture for phosphofructokinase (31) in order to inhibit fructose diphosphatase which competes with aldolase for fructose-1,6-bisphosphate. Hence, measurements made with crude extracts without 5′-AMP in the assay mixture give only a qualitative indication of the activity of the enzyme under a particular set of conditions because such measurements could reflect changes in fructose diphosphatase activity. Nevertheless, the greatest differences between

control and dibutyryl cyclic AMP-treated hepatocytes are observed without 5′-AMP in the assay mixture and it does not appear, from direct measurements of the enzyme, that changes in activity of fructose diphosphatase are involved. Our working hypothesis is that cyclic AMP causes an alteration in phosphofructokinase which makes it more responsive to 5′-AMP activation or 5′-AMP reversal of ATP inhibition.

It is clear that the difference in activity between control and dibutyryl cyclic AMP-treated hepatocytes is greater at low than at high fructose-6-phosphate concentrations. As such, this differs from the preliminary report of Cook et al. (32) of a change in V_{max} of phosphofructokinase in mouse liver perfused with glucagon. It also differs from the apparent change in V_{max} reported by Brand et al. (33-35) to be caused by phosphorylation of the enzyme. Our results agree, however, with the change in $S_{0.5}$ for fructose-6-phosphate reported to occur in response to glucagon or dibutyryl cyclic AMP by Pilkis et al. (17), Castano et al. (18) and Clark et al. (19). Castano et al. (18) established that the changes in enzyme activity caused by glucagon persisted even after partial purification of the enzyme by Sephadex G-25 and agarose-ATP chromatography. Thus, this study confirms those of Pilkis et al. (17), Castano et al. (18) and Clark et al. (19) and makes the important points that ITP is a useful substitute for ATP as the phosphate donor in the assay of phosphofructokinase activity in crude extracts and that no corresponding change in activity of fructose diphosphatase is involved.

Kagimoto and Uyeda (36) recently reported glucagon stimulation in vivo of labeled inorganic phosphate incorporation into rat liver phosphofructokinase. However, the activity difference for phosphofructokinase found, contrary to that reported here and by others (17-19), was manifested as an increase in the sensitivity of the enzyme to ATP inhibition. ATP inhibition of the enzyme is known to be reversed by 5′-AMP and it is reported here, as well as by Pilkis et al. (17), that including 5′-AMP in the assay largely abolishes the difference in activity of the enzyme extracted from control and cyclic AMP-treated cells. Thus, the results of Kagimoto and Uyeda (36) may merely reflect the different conditions chosen to assay the enzyme. To be consistent, however, the cyclic AMP-inactivated enzyme would have to be sensitive to ITP inhibition, in contrast to the native enzyme (28), and 5′-AMP would have to reverse that inhibition. This is under investigation.

Although the available evidence strongly argues that we are dealing with a cyclic AMP-dependent interconversion of phosphofructokinase between active and inactive forms by the phosphorylation-dephosphorylation mechanism, additional studies are clearly needed to define the link to the cyclic AMP system. Regardless of the exact mechanism, rapid inactivation of phosphofructokinase by a cyclic AMP-dependent mechanism can account in large part for cyclic AMP inhibition of hepatic glycolysis and lipogenesis.

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Effect of Dietary Fatty Acid Composition on the Biosynthesis of Unsaturated Fatty Acids in Rat Liver Microsomes

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ABSTRACT

A study was made of the influence of semisynthetic diets of low and high unsaturation on the fatty acid composition and desaturation-chain elongation enzymatic activity of the liver microsomal fractions of male Sprague-Dawley rats of different ages. Groups of rats were fed 5 or 20% coconut oil (CO), or a 5 or 20% mixture of corn and menhaden oils (3:7) (CME) from weaning to 100 wk of age. Growth rate and food consumption were measured during this period in which animals were sacrificed at 36, 57, 77 and 100 wk of age. Both the level and composition of the dietary fat supplements produced marked effects on the fatty acid composition of the liver microsomal lipids. In general, the fatty acid composition of the microsomal fractions reflected that of the dietary fat and was more unsaturated with the higher level of fat fed. The rate of conversion of linoleic to arachidonic acid in assays performed in vitro with liver microsomal preparations from animals of the different groups also showed marked differences. The 6-desaturase-chain elongation activity was higher in the 5% than 20% group and corresponded to the essential fatty acid (EFA) status of the animals in these groups as represented by the triene-tetraene ratio of the microsomal lipid. The relationship of the 6-desaturase activity to fatty acid composition of the microsomal lipid indicated that if varied directly with the level of 20:3ω9, 18:1 and 16:1 and was inhibited by arachidonic acid. The activity of the 6-desaturase enzyme system was lowest in the liver microsomal fraction obtained from the animals fed the CME diets and appeared to be suppressed by the high levels of 20:5 and 22:6 that accumulated in the microsomal lipid. Accordingly, the levels of arachidonic acid were lower in the microsomal lipid of these groups than those of the corresponding CO groups in spite of a greater abundance of linoleic acid in the diet. The data suggest that the activity of the 6-desaturase-chain elongation system is regulated by the fatty acid composition of the microsomal lipid as influenced by the composition of the dietary fat.

INTRODUCTION

Although pathways in the biosynthesis of common unsaturated fatty acids have been elucidated (1,2), the influence of dietary fatty acids on tissue fatty acid composition is highly complex and not well defined. The control of unsaturated fatty acid biosynthesis is believed to reside mainly in the regulation of microsomal desaturase activity that occurs at the 6 and 9 positions in the fatty acid chains (3-7). Desaturation at these positions, as well as at the 5 position, is believed to be catalyzed by specific enzymes (3-5) and their regulation appears to involve the electron transport system (8-11). They are subject to diurnal changes but the rhythm of these changes could not be associated with any particular biochemical parameter (12). Under certain conditions, desaturases respond to hormonal stimuli (13-18), fasting and refeeding regimens (13,19,20), high levels of dietary carbohydrate (13,19,21) or protein (13,15,16,21) and an essential fatty acid (EFA) deficiency (6,21-24). Holloway and Holloway (7,25) considered that alteration of stearoyl-CoA desaturase activity in an EFA deficiency might result from changes in membrane fluidity brought about by changes in composition of the lipid. Their studies did not confirm nor rule out this possibility, although the importance of protein synthesis in the activity of this desaturase, as reported by Oshino and Sato (26) and Strittmatter et al. (27) was demonstrated. Peluffo et al. (28) found that changes in 6-desaturase activity appeared to correlate with the phospholipid-triglyceride ratio of the liver microsomal fraction of rats fed an EFA-deficient diet and suggested that the activity of this enzyme system might be related to fluidity of the membrane. Accordingly, it was proposed that the increase in 6-desaturase activity in EFA deficient animals was a response to maintain fluidity of the membrane by regulation of the ratio of saturated-to-unsaturated fatty acids.

The competitive nature of reactions involved in the biosynthesis of unsaturated fatty acids have been well demonstrated by in vitro (4,28-31), as well as in vivo (32-36) experiments. Sprecher (4) determined the relative rates of desaturation and chain elongation in the interconversion of unsaturated fatty acids in vitro and suggested that tissue fatty acid

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composition is determined on the basis of the relative rates of these reactions and those of retroconversion and the incorporation of fatty acids into lipids.

In this study, relationships between the composition and level of dietary fat, fatty acid composition of liver microsomal lipids and the interconversion of linoleate in vitro were investigated in rats fed diets containing varying levels of linoleic and polyunsaturated fatty acids.

EXPERIMENTAL

Animals

Weanling male Sprague-Dawley rats purchased from Dan Rolfsmeyer Co., Madison, WI, were divided into 4 groups of 15 animals and fed a basic fat-free diet supplemented with 5 or 20% by wt of coconut oil (CO) or a 3:7 mixture of corn and menhaden oils (CME). The diets, the composition of which is shown in Table I, were made isocaloric by adjusting the relative amounts of fat, carbohydrate and fiber. The animals were weighed at 2-day intervals; food consumption was measured daily on 5 animals of each group.

Animals were sacrificed at 36, 57, 77 and 100 wk of age by exsanguination under a light ether anesthetic. Various tissues and organs were weighed, quick frozen and stored at -70 C for studies that will be reported at a future date.

Preparation of Liver Microsomes

Freshly excised livers were perfused with saline and homogenized in a Potter-Elvehjem homogenizer in 2 vol of a solution containing 0.25 M sucrose, 5 mM $MgCl_2$, 0.15 M KCl, 1.5 mM GSH and 50 mM of potassium phosphate buffer, pH 7.0. Homogenates were centrifuged at 800 x g for 10 min to remove cell debris, then at 10,000 x g for 20 min. The microsomal fraction was recovered by centrifuging the 10,000 x g supernatant at 100,000 x g for 1 hr. The pellet was suspended in a solution containing 5 mM $MgCl_2$, 0.15 M KCl, 1.5 mM GSH and 50 mM potassium phosphate buffer (pH 7.0) to give a final concentration of 20 mg per ml protein, determined by the Lowry et al. method (37).

Incubation Conditions

ATP, CoASH, malonyl-CoA, NADH and bovine serum albumin, containing less than 0.005% fatty acid, were purchased from Sigma Chemical Co., St. Louis, MO; GSH was purchased from P-L Biochemicals, Inc., Milwaukee, WI; [1-^{14}C]linoleic acid (56 μCi/mol) was purchased from New England Nuclear, Boston, MA, purified by thin layer chromatography (TLC), converted to its ammonium salt and bound to bovine serum albumin (38). All incubations were carried out at 37 C in a total vol of 2.0 ml. For measurement of desaturase activity, each incubation was carried out for 10 min in the medium containing the following: 10 μmol $MgCl_2$, 0.3 mmol KCl, 3 μmol GSH, 10 μmol ATP, 0.6 μmol CoA, 2.5 μmol NADH, 100 μmol potassium phosphate buffer, pH 7.0, 200 nmol radioactive linoleic acid and

TABLE I

Diet Composition

	5% Fat diet (% by wt)	20% Fat diet (% by wt)
Casein (vitamin test)	22.50	22.50
L-Cystine[a]	0.20	0.20
Wesson salt mixture plus $ZnCl_2$ and $MnSO_4 \cdot H_2O$[b]	4.03	4.03
Choline mix[c]	1.00	1.00
Vitamin mix[d]	1.00	1.00
Cellulose (Alphacel)	10.25	29.00
Sucrose	56.02	22.27
Fat	5.00	20.00

[a]L-Cystine is added to the diet to bolster the level of sulfur-containing amino acids.

[b]Wesson salt mixture does not contain zinc or manganese, hence these elements are added to the mix as follows: 0.60 g $ZnCl_2$ and 0.90 g $MnSO_4 \cdot H_2O$/200 g salt mixture.

[c]Choline mix consists of 22% choline dihydrogen citrate in vitamin test casein.

[d]Vitamin mix (1 kg) contains: 2.5 g thiamine HCl, 2.5 g riboflavin, 9.0 g nicotinic acid, 9.0 g calcium pantothenate, 2.0 g pyridoxine HCl, 4.0 g cyanobalamin (B_{12}), 7.5 g *p*-aminobenzoic acid, 0.1 g folic acid, 0.02 g biotin, 20.0 g *meso*-inositol, 0.5 g menadione (vitamin K), 943.0 g vitamin test casein. Fat and vitamins A, D and E are mixed into the diet daily and stored at 0 C overnight. Vitamin D_2, 5.0 mg; retinol acetate, 6.9 mg; α-tocopherol acetate, 300 mg/kg of diet.

5 mg of microsomal protein in 0.001% triton X-100.

The same conditions were used for determination of desaturation-chain elongation reactions except that 0.6 μmol malonyl CoA was added to the incubation mixture.

Incubations were stopped by the addition of 10 ml dimethoxypropane containing 200 μl concentrated HCl to 1 ml of the incubation medium. After a reaction time of 20 min to allow for conversion of the water to methanol and acetone, these solvents and the excess DMP were evaporated in a stream of nitrogen at room temperature, and the lipid interesterified with methanol as described by Shimasaki et al. (39).

Radioactivities of the methyl esters were determined on fractions isolated by gas liquid chromatography (GLC) using an Aerograph Model 600-D gas chromatograph equipped with a 9:1 splitter, and a 12' x 0.124" id column packed with 10% Silar 10C on 100-200 mesh Gas-Chrom Q at 210 C with a flow rate of N_2 of 20 cc/min. Fractions (peaks in the chromatogram) corresponding to each fatty acid ester were collected in glass tubes attached directly to the outlet of the splitter, coincident with their detection by the flame detector, and transferred to scintillation counting vials by washing the tubes with 15 ml scintillation fluid (5.5 g Permablend I/liter toluene). Radioactivity was counted in a packard Model 3310 liquid scintillation spectrometer. Recovery of radioactivity by this technique of collection was ca. 70%. The activity of 6-desaturase, determined independently, was calculated from the counts of 18:3 corrected for background. The activities of 6-desaturase, chain elongation and 5-desaturase in the overall reaction were determined simultaneously from the total counts of the products corrected for background, the proportion of the counts in the 20:3 + 20:4 acids and the 20:4 fraction, respectively. In the assay conditions described for 6-desaturase activity, the amount of 18:3 formed was proportional to reaction time for ca. 15 min and the resulting rates were proportional to protein concentration. In the assay of chain elongation, after a short induction period in which 18:3 was formed, the rate of formation of 20:3 and 20:4 was constant also for a period of ca. 15 min. These results and the good agreement between 6-desaturase activity determined independently and from the overall reaction assured the validity of the enzyme assay conditions.

Fatty acid composition of liver microsomal lipid was determined on methyl esters prepared by interesterification with HCl as a catalyst (39) using a Hewlett Packard Model 5840A gas chromatograph. This analysis was also carried out with a 12' x 0.125" id column packed with Silar 10C on 100-200 mesh Gas-Chrom Q at 200-250 C programmed at 2.0 C/min with a flow rate of nitrogen at 10 cc/min.

RESULTS

Although there were large differences in fatty acid composition of the 2 dietary fats employed and the amounts of individual fatty acid consumed (Table II), there were no significant differences in growth rate of the animals or food consumption (Fig. 1) in the different groups.

The amount of linoleic acid consumed and the percentage of this fatty acid of the dietary calories varied greatly from group to group, as shown in Table II. The percentage of the dietary calories of this fatty acid in the 5% CO diet was less than that considered the normal requirement of EFA, whereas that in the other diets was more than adequate. In addition to supplying an adequate level of linoleic acid, the CME supplement contained relatively large amounts of 20:5 and 22:6 acids.

Fatty acid composition analyses of the liver microsomal lipid showed no significant differences within any one group at different ages of the animals from 36 to 100 wk. Thus, all of the values obtained over this period for each group were combined to obtain a representative fatty acid analysis of the microsomal lipid for each group, as shown in Table III.

These analyses showed that both the level and composition of the fat supplements produced significant differences in the fatty acid composition of the liver microsomal lipids. In spite of the fact that the animals of the 20% CO group consumed a much larger amount of fat (Table II), the levels of palmitoleic (16:1) and oleic (18:1) acids were significantly lower in this group than in the 5% CO group, and these differences were not compensated by differences in the fat content of the microsomal fraction. As expected, the levels of both linoleic and arachidonic acids were higher in the 20% than in the 5% CO group. The 5% group contained an elevated level of 20:3ω9 and, accordingly, the triene/tetraene ratio was elevated compared to that of the 20% CO group. This observation is in accord with the fact that the animals of the 5% CO group received only 0.38% of their dietary calories as linoleate compared to 1.52% for the 20% CO group (Table II).

The fatty acid composition of the liver microsomal lipid of the animals fed the mixture

TABLE II

Consumption and Composition of Dietary Fatty Acids

Fatty acid composition of dietary fat[a] (% wt)	Coconut oil (CO) diet					Corn-menhaden oil (CME) diet				
	Fat consumption					Fatty acid composition of dietary fat[a] (% wt)	Fat consumption			
	5% supplement		20% supplement				5% supplement		20% supplement	
	% dietary calories	g/wk[b]	% dietary calories	g/wk			% dietary calories	g/wk	% dietary calories	g/wk
10:0	4	0.50	0.28	2.00	1.12	–	–	–	–	–
12:0	48	6.00	3.36	24.00	13.44	–	–	–	–	–
14:0	21	2.63	1.47	10.50	5.88	8	1.00	0.56	4.0	2.24
16:0	11	1.38	0.77	5.5	3.08	19	2.38	1.33	10.0	5.32
16:1	–	–	–	–	–	11	1.38	0.77	5.5	3.08
18:0	3	0.38	0.21	1.52	0.84	4	0.50	0.28	2.01	1.12
18:1	9	1.13	0.63	4.50	2.52	17	2.13	1.19	8.5	4.76
18:2	3	0.38	0.21	1.52	0.84	20	2.50	1.40	10.0	5.60
20:0	–	–	–	–	–	2	0.25	0.14	1.0	0.56
20:5	–	–	–	–	–	12	1.50	0.84	6.0	3.36
22:6	–	–	–	–	–	7	0.88	0.49	3.5	1.96

[a]Shorthand designation for fatty acids, number before colon = chain length; number after colon = number of double bonds.

[b]Based on food consumption of 20 g/day.

FIG. 1. Growth rate and food consumption of animals. 5% Coconut oil (CO) diet = A and ●—●; 20% coconut oil (CO) diet = B and ○—○; 5% corn-menhaden oil (CME) diet = C and ▲—▲; 20% corn-menhaden oil (CME) diet = D and △—△.

of corn and menhaden oils differed considerably from those of the CO groups and the differences were reflected in the fatty acid composition of the dietary fat, as shown in Table III. A major difference in the fatty acid composition of the CO and CME fat supplements was in the content of 20:5 and 22:6. Correspondingly, larger amounts of these fatty acids accumulated in the liver microsomal lipids of the CME groups. However, in spite of the fact that much larger amounts of these acids were consumed by the 20% than the 5% CME group, the levels of these acids in the microsomal lipid were not significantly different in the 2 groups. Another apparent anomaly was in the levels of 16:1 and 18:1, which were higher in the 5% than the 20% CME group, inasmuch as the animals in the 20% CME group consumed much larger amounts of these acids. The levels of 18:2 and 20:4 were slightly higher in the 20% than the 5% CME group as might be expected because the 20% CME group consumed ca. 4 times more linoleate than the 5% CME group. In fact, the level of arachidonic acid in the 20% CME group was not significantly different from that of the 5% CO group which exhibited a borderline EFA deficiency as indicated by the triene/tetraene ratio and the elevated levels of 16:1 and 18:1. Further, the level of arachidonic acid in the 5% CME group was even lower than that of the 5% CO group, 8.6% vs 11.5%. There was no 20:3ω9 in the microsomal lipid or any other evidence of an EFA deficiency in the animals of the 5% CME group.

The 6-desaturase enzyme activity, determined independently, and the overall desaturase-chain elongation enzyme activity were significantly greater in the 5% than in the 20% CO group which in turn was significantly

TABLE III

Fatty Acid Composition of Liver Microsomal Lipid (% wt)

Dietary group (fat supplement)[a]	5% CO	20% CO	5% CME	20% CME
Microsomal fatty acid content (mg/mg protein)	0.210 ± 0.048	0.271 ± 0.035	0.225 ± 0.045	0.246 ± 0.063
Fatty acid composition[b]				
14:0[c]	1.1 ± 0.3	2.1 ± 0.3	–	–
16:0	20.1 ± 1.4	19.6 ± 1.2	25.9 ± 2.5	22.1 ± 1.6
16:1	7.2 ± 1.5	3.4 ± 0.6	5.4 ± 1.4	2.6 ± 0.2
18:0	17.3 ± 1.5	19.5 ± 1.4	15.0 ± 1.3	17.8 ± 1.6
18:1	20.6 ± 1.8	14.6 ± 1.7	10.2 ± 0.7	6.8 ± 0.6
18:2	4.1 ± 0.8	7.4 ± 1.5	8.8 ± 0.8	13.9 ± 0.9
20:3ω9	8.7 ± 2.1	2.1 ± 1.1	–	–
20:3ω6	2.2 ± 0.2	2.3 ± 0.2	1.2 ± 0.1	1.4 ± 0.2
20:4	11.5 ± 0.4	20.8 ± 2.5	8.6 ± 0.8	11.6 ± 1.4
20:5	–	–	8.6 ± 1.4	7.3 ± 1.4
22:4	2.0 ± 0.5	2.3 ± 1.2	–	–
22:5	–	–	2.3 ± 0.3	1.6 ± 0.2
22:6	1.3 ± 0.3	2.4 ± 0.8	11.5 ± 2.6	12.1 ± 2.2
20:3ω9/20:4	0.76 ± 0.2	0.10 ± 0.07	–	–

[a]CO = coconut oil, CME = corn-menhaden oil mixture (3:7).

[b]M ± SD, n = 8. The values in the boxes are significantly different ($P < 0.001$).

[c]Shorthand designation of fatty acid. Number before colon = chain length, number after colon = number of double bonds.

greater than those of the 5% CME and 20% CME groups, as shown in Table IV.

These analyses (Table IV) were calculated from the combined values determined in animals of 36-77 wk of age. The values were combined because, as with fatty acid composition, no significant differences were observed over this period of time within any of the individual groups.

DISCUSSION

This study shows that both the level and fatty acid composition of the dietary fat produced marked effects on the composition and biosynthesis of unsaturated fatty acids in the liver. The 5% CO diet used in this study contained less linoleate than that considered adequate to satisfy the EFA requirements of the rat (39-41). Accordingly, the biochemical parameters generally associated with an EFA deficiency were observed with this diet, namely an increase in the levels of 16:1, 18:1 and 20:3ω9, and a high triene-tetraene ratio. Also, desaturase activity was elevated in the liver microsomal fraction as observed by others in animals with an EFA deficiency (6,24,25). The animals of this group showed no dermal symptoms of an EFA deficiency and the liver microsomal lipid contained significant levels of linoleic (4.1%) and arachidonic (11.5%) acids. Nevertheless, the conditions in the animals of this group favored enhancement of the activity of the 6-desaturase-chain elongation enzyme system and some oleic acid was converted to 20:3ω9. The activity of 9-desaturase was also elevated in the animals of the 5% CO group as evidenced by the high levels of 16:1 and 18:1 in the microsomal lipid. Apparently, the same conditions that induced an elevation of 6-desaturase activity also favored 9-desaturase activity. Comparison of the 5% and 20% CO groups showed that when the amount of linoleate consumed was increased from 0.38% to 1.52% of the dietary calories, the amount deposited in the microsomal lipid was increased from 4.1% to 7.4%. The level of arachidonic acid was increased from 11.5% to 20.8% in the microsomal lipid of the 20% CO animals in spite of the fact that the activity of the 6-

TABLE IV

Liver Microsomal Enzyme Activities (nmol/min/mg protein)

Dietary group[a] (% fat supplement)	5% CO	20% CO	5% CME	20% CME
6-desaturase[b]	0.460 ± 0.10[c]	0.256 ± 0.052	0.128 ± 0.028	0.166 ± 0.038
6-desaturase chain elongation	0.404 ± 0.066	0.284 ± 0.048	0.162 ± 0.014	0.182 ± 0.040
5-desaturase	0.252 ± 0.052	0.178 ± 0.036	0.086 ± 0.012	0.094 ± 0.018
	0.098 ± 0.022	0.056 ± 0.010	0.020 ± 0.008	0.022 ± 0.008

[a]CO = coconut oil; CME = corn-menhaden oil mixture (3:7).

[b]Determined separately.

[c]M ± SD, n = 6. 5% CO vs 20% is significantly different, $P < 0.01$; 5% CME is significantly different than 5% CO and 20% CME, $P < 0.001$; 20% CME is significantly different than 5% CO and 20% CO groups, $P < 0.001$ and $P < 0.01$, respectively. The values for 20% CME and 5% CME are not significantly different.

desaturase-chain elongation enzyme system was lower. Apparently, with the consumption of a larger amount of linoleic acid, more becomes available for conversion to arachidonic acid inasmuch as its incorporation into lipid is a competing reaction (29). On the other hand, the decreased level of 20:3ω9 appears to be related to the decreased activity of this enzyme system. The decreased 20:3ω9 also might be attributed in part to the low level of oleic acid, its precursor, as a result of an inhibitory action of arachidonic acid on 9-desaturase activity. Accordingly, the dramatic effect of the decrease in 20:3ω9 upon switching EFA-deficient animals to a diet containing linoleic acid might not result so much from substrate competitive inhibition as the suppression of 6- and 9-desaturase activity by arachidonic acid. That substrate competitive inhibition might not be the major in vivo controlling mechanism in the synthesis of 20:3ω9 also has been suggested by Sprecher (4). Early in vitro assay studies indicated that arachidonic acid enhanced 6-desaturase activity (28,41). However, later work (5) showed that it does inhibit 6-desaturase activity in accordance with the hypothesis just stated.

Examination of the fatty acid composition of the liver microsomal lipid relative to the 6-desaturase-chain elongation activity of the CME groups indicated that the biosynthesis of unsaturated fatty acids is related to the overall polyunsaturation of the microsomal lipid. The major difference in the fatty acid composition of the microsomal lipid of the CME and CO groups is the high content of 20:5 and 22:6 acids in the former. These acids originate primarily from the diet inasmuch as only trace amounts were found in the animals fed the CO supplement, and the CME supplement did not contain any acids that might serve as their precursors. Thus, the relatively low concentration of arachidonic acid and the high ratio of linoleic-to-arachidonic acid in the CME groups appear to result from an inhibitory effect of the 20:5 and 22:6 acids of the microsomal lipid on the 6-desaturation-chain elongation enzyme system. It appears that the polyunsaturation of the microsomal lipid exerts a controlling effect on the 6-desaturase-chain elongation activity regardless of whether it accumulates from the diet, as in the case of 20:5 and 22:6, or as a product of the reaction, as with arachidonic acid. These observations also indicate that the influence of the microsomal fatty acid composition on 6-desaturase-chain elongation activity is a major factor in the control of fatty acid biosynthesis as opposed to substrate competitive inhibition.

It is notable that the 20:5 and 22:6 accumulated in the microsomal lipid to approximately the same levels in both the 5% and 20% CME groups in spite of the fact that the animals of the 20% CME group consumed much larger amounts of these fatty acids. Obviously, the deposition of these fatty acids in the liver microsomal fraction is under metabolic control which, under different conditions, might influence the activity of the 6-desaturase-chain elongation enzyme system.

Another interesting observation relative to the fatty acid composition of the microsomal lipid of the CME groups is the higher content of 16:1 and 18:1 in the animals of the 5% group compared to the 20% group, particularly since the animals in the 20% group consumed much larger amounts of these fatty acids. The lower content of these fatty acids in the 20% CME group does not appear to result from an inhibition of the 9-desaturase activity by the polyunsaturated fatty acids of the microsomal lipid because there is little difference between the fatty acid composition of these acids in the 2 CME groups. Both 16:1 and 18:1 acids originate, in part, from the diet and apparently are subject to metabolic control as noted for

the 20:5 and 22:6 acids. Thus, the lower levels of 16:1 and 18:1 in the 20% CME group compared to the 5% CME group might result from a form of metabolic control by the large amounts of these acids supplied exogenously, just as lipogenesis is decreased by high-fat diets (42).

Regardless, this study shows that the type and amount of fat in the diet exerts a profound influence on the composition of the liver microsomal lipid and that a primary factor in the regulation of the biosynthesis of unsaturated fatty acids is the inhibitory effect of polyunsaturated fatty acids on desaturation-chain elongation enzyme activities. The mechanism by which microsomal fatty acid composition functions to control desaturation-chain elongation activity is unknown. However, the relative rates of the sequential reactions in the conversion of linoleic acid to arachidonic acid are influenced by all of the diets in the same pattern. Hence, it appears that the dietary fats influence the enzyme system in the conversion of linoleic to arachidonic acid in the same way as might be expected by an effect on a common property such as fluidity or other phenomenon associated with the microsomal membrane to which these enzymes are bound.

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The Influence of Urea on Lipogenesis in Renal Papillae of Rats

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ABSTRACT

The osmolality has been determined for the papillary interstitial fluids obtained from the isolated papillae of rats in different states of water balance. Hypertonic buffers for in vitro experiments were prepared by addition of urea to regular Krebs-Ringer phosphate buffers (340 mosmol/kg H_2O) since urea is the most changeable solute of the renal papillary tissue in response to external influences. The effect of such hypertonic buffers on papillary lipogenesis was very pronounced. The fatty acid synthesis from acetate decreased by a factor of 7-9 in response to increased osmolality from 340 to 1370 mosmol/kg H_2O. In the physiological range of osmolalities (500-1800 mosmol/kg H_2O), the de novo synthesis of papillary glycerolipids from glucose decreased by a factor of ca. 5. A possible specific inhibitory effect of hypertonic buffers on the pentose phosphate cycle was studied with a negative result. It is concluded that the addition of urea causes a decrease of total energy metabolism in the tissue.

INTRODUCTION

An exchange of acyl groups between glycerolipids of the renal papilla of rats has been demonstrated in vitro (1,2). Leucocytes display the same phenomenon during phagocytosis (3) and phospholipid acyl groups can apparently be shifted to triacylglycerols in L mouse fibroblasts and vice-versa depending on the availability of exogenous fatty acids (4). The interstitial cells of the renal papilla, which contain the papillary triacylglycerols as droplets (5), resemble in some ways both cell types. Like fibroblasts, the interstitial cells are of mesenchymal origin and like leucocytes, a few interstitial cells showed phagocytic properties after induced water diuresis in rats in contrast to interstitial cells in rats under normal states of hydration and dehydration (6). Furthermore, radioactive acetate incorporation studies in rat leucocytes show that exactly the same spectrum of radioactive fatty acids is synthesized as in the renal papilla with docosatetraenoic acid as the predominant radioactive acid (7). An increased osmolality inhibits the phagocytosis of leucocytes in vitro (8). Therefore, these observations prompted us to study the lipogenesis in hypertonic media. If the observed exchange of fatty acids in the renal papilla is correlated with the phagocytic properties of the interstitial cells and thereby with the osmolality of the papillary interstitium, then the phenomenon should disappear when the osmolality increases.

MATERIALS AND METHODS

All radioactive compounds were obtained from the Radiochemical Centre, Amersham, England. Sprague-Dawley rats (250 g) were divided into 3 groups. Group I: (normal rats) rats with free access to water; group II: (saline-loaded rats) rats with only 1% NaCl-solution to drink for 48 hr; group III: (water-deprived rats) rats deprived of water for 24 hr.

Incubation of Renal Papillae

The rats were decapitated and the renal papillae were excised, sliced and incubated for different periods of time in mediums based on Krebs-Ringer phosphate buffer (pH = 7.4, 340 mosmol/kg H_2O) with 5 mM glucose and radioactive acetate and glucose as described previously (9,10). Oxygen consumption was measured every 30 min to control the normal function of the slices during incubation. Media of higher osmolality were prepared by adding urea.

Measurement of Tissue and Medium Osmolality

Measurement of tissue osmolality was done by a modification of the technique described by Lee et al. (11).

The isolated papilla (in many experiments the contralateral to the one incubated) was placed under 0.2 ml paraffin oil (specific gravity 0.88) in a pointed centrifuge tube (80 mm x 5 mm id) provided with a small hole (0.2 to 0.4 mm in diameter). This tube was placed in a second tube (95 mm x 13 mm id) containing 2 ml of paraffin oil and fixed at the top with a plastic rim. Centrifugation for 20 min at 3000 rpm caused a fluid fraction (ca. 300-400 nl) to separate from the tissue in the bottom of the second tube; 0.2-0.4 nl of this fluid was measured. The real osmolality was obtained from this freezing-point depression according to the Scientific Tables (12).

The osmolality of urine samples and of

incubation media also was measured by freezing point-depression.

Lipid Extraction and Analyses

Lipids were extracted with a methylene chloride/methanol mixture (2:1 by vol). Separation of lipid classes as well as separation within classes was done by thin layer chromatography (TLC) (15). Transesterification of major lipids (triacylglycerols and phospholipids) was carried out in super dried methanol containing 5% gaseous HCl as described previously (5) and the resulting methyl esters were analyzed by radio gas liquid chromatography (GLC). Quantification of triacylglycerols and phospholipids was performed by glycerol and phosphorus analyses after transesterification according to Hanahan and Olley (13) and Bartlett (14), respectively.

RESULTS

Acetate Incubations

The papillary fatty acid synthesis was studied in a few experiments with [1-^{14}C]-acetate as substrate and medium osmolality of 1370 mosmol/kg H_2O. The rate of incorporation of acetate into fatty acids of the papillary glycerolipids goes down by a factor of 7-9 (Fig. 1).

Radio-GLC of the fatty acids of triacylglycerols and phospholipids revealed that the de novo synthesis of fatty acids decreased relatively more than chain elongation of preexisting fatty acids.

Previous experiments with [^{14}C] acetate and [^{14}C] glucose revealed an initial preferential incorporation of 7,10,13,16-docosatetraenoic acid (22:4) into papillary phospholipids relative to that into triacylglycerols. After ca. 6 hr of incubation, the acid had roughly the same specific activity (sp act) in lipid droplet triacylglycerols and in membrane phospholipids (Fig. 6 in ref. 2). This initial preferential incorporation of 7,10,13,16-docosatetraenoic acid into phospholipids could not be demonstrated at high osmolality since the sp act of the acid in the 2 lipid classes was the same independent of incubation time. Thus, the exchange of polyunsaturated fatty acids between papillary triacylglycerols and phospholipids observed after incubation in isosmotic media could not be demonstrated in hyperosmotic media.

Osmolality Measurements

The osmolality of renal papillary interstitium can vary quite considerably in response to external influences, largely because of the papillary urea content. It was desirable to know the exact extracellular osmolality in the papillae before they were used for experiments. Therefore, the osmolality of the fluids which could be isolated from papillae by centrifugation was measured as described in Methods. Determinations were carried out on papillary fluids isolated from rats in 3 different states of water balance (Table I). The osmolality of the fluids obtained from the papillary tissue depended on the positioning of the papillae during centrifugation, so that with the tip of the papillae downward a fluid was released with higher osmolality than with the reverse positioning. This result was to be expected on the basis of the known osmotic gradient through the papilla of rat kidney during the normal state of hydration (15). The osmolality of the fluid derived from the base of the papillae will be very near the average osmolality of the largest part of the papillary interstitium. The value obtained in normal rat papillae (814 mosmol/kg H_2O) is close to the approximate osmolality as estimated by Law (16) (740

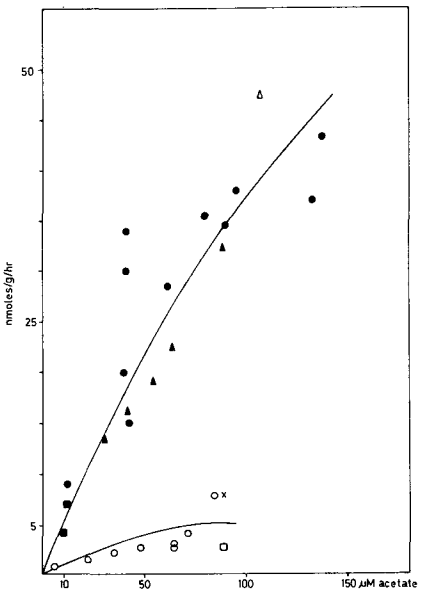

FIG. 1. The relationship between the in vitro rate of [1-^{14}C] acetate incorporation into fatty acids of papillary lipids and the acetate concentration at 2 different osmolalities. Papillary slices (50 mg) randomly selected from 4 different rats were incubated with [^{14}C] acetate for different periods of time in 2 ml buffer solution without and with 2.06 mmol urea. Data for different incubation periods at 340 mosmol/kg H_2O (▲ = 1 hr; ● = 2 hr; ■ = 4 hr; and △ = 7 hr) and at 1370 mosmol/kg H_2O (x = 1 hr; 10 = 2 hr; and □ = 4 hr). Each value represents one incubation experiment.

TABLE I

Osmolality of Papillary Fluids Obtained by Centrifugation from 3 Different Groups of Rats in Different States of Water Balance[a]

Group I		Group II		Group III			Eviscerated rats	
mmol/kg H_2O ± SE		mmol/kg H_2O ± SE		mmol/kg H_2O		Diuresis	mmol/kg H_2O	
A(n = 7)	B(n = 4)	A(n = 12)	B(n = 5)	A	B	ml/48 hr	A	B
1409 ± 105	814 ± 52	1905 ± 124	885 ± 39	1910	784	21	697	514
				1462	751	30	400	444
				1402	675	38	428	395
				1505	-	44	589	498
				1533	711	56	-	-
				1076	640	64	-	-
				980	-	72	-	-
				592	-	74	-	-

[a]See Materials and Methods for description of conditions. Whole papillae were placed under paraffin oil (spec. grav. 0.88) in the inner of a double centrifuge tube provided with a small hole. In A the tip and in B the base of the papilla faced the hole. The freezing point depression of the fluid collected in the outer tube was measured. The osmolality for groups I and II was measured in 1 kidney papilla, whereas the other was used for incubations. In 5 animals of group III, A and B data are from the same rat. For calculating the osmolality from the freezing-point depression, see ref. 12.

mosmol/kg H_2O) on the basis of tissue analyses and expressed as the sum of the concentrations $2([Na^+] + [K^+]) + [urea]$. On the other hand, the osmolality of the papillary fluids isolated from the tip of papillae was not the same as that of urine derived from the bladder at the moment of decapitation. Analyses of urine from groups I and III rats in all cases gave a urine with a much higher osmolality than the fluid derived from the tip of the corresponding papilla. In contrast, the urine from rats in group II had the same or a slightly lower osmolality than the fluid isolated from the tip of the papilla.

The standard error of the mean (SEM) was less than 10% in groups I and III whereas the osmolality determinations of papillary fluids obtained from NaCl-loaded rats was correlated to the diuresis obtained (Table I, column 3).

Incubation with Glucose

Papillae isolated from the already mentioned 3 groups of rats form the basis for the in vitro study of the papillary lipogenesis from [U-^{14}C] glucose.

Incubation of papillary slices for 2-4 hr with radioactive glucose as lipogenic precursor resulted in labeled glycerolipids which constitute 85% or more of total radioactive lipids independent of medium osmolality. The largest fraction of the radioactivity in these lipids was present in the glycerol backbone. After incubations in isosmotic media the glycerol moiety contained 82.2% ± 1.4% (n = 9) in phospholipids and 73.7% ± 3.0% (n = 7) in triacylglycerols. These percentages increased gradually when the tonicity of the media was increased by means of urea additions so that at 750 mosmol/kg H_2O they are 96% and 93%, at 1000 mosmol/kg H_2O 97.5% and 96.5% and from 1800 mosmol/kg H_2O and upwards 99.5% and 99.0%, respectively. This means that the synthesis of fatty acids from glucose via acetyl-CoA decreases by a factor of 10 when the osmolality is increased from 400 to 1800 mosmol/kg H_2O. This is in accordance with the observation already given that the lipogenesis from acetate is decreased by a factor of 7-9 by varying the medium osmolality from 340 to 1370 mosmol/kg H_2O and demonstrates that the acetate equivalence of glucose does not change with the osmolality.

Papillary slices from normal rats incorporate 354 nmol glucose/g papillary tissue wet wt/hr ± 36 (n = 12) when medium osmolality is 340 mosmol/kg H_2O and glucose concentration 5 mM. However, the synthesis of the complex lipids is highly dependent on medium urea concentration (Fig. 2). The synthesis decreases with a factor of more than 5 when the osmolality is increased within the physiological range of osmolalities 500-1800 mosmol/kg H_2O. If the natural logarithm of medium osmolality is plotted as a function of the rate of glucose incorporation into papillary lipids, a correlation between the 2 variables is found with a coefficient of correlation 0.88 (Fig. 2). The possibility exists that the observed behavior is merely a consequence of an adaptation, when papillae of a certain osmolality are transferred to a medium of quite a different osmolality. However, the curves are identical for the 3

groups of rats and no minimum around zero was found by plotting the incorporation rate as a function of the change in osmolality (medium osmolality -osmolality of fresh tissue fluid).

^{14}C Glucose Oxidation

It is known from the Bernanke and Epstein study (17) and from that of Kean et al. (18) that a considerable part of the glucose taken up by the renal papillae of dog is metabolized through the pentose phosphate cycle. In agreement with these studies in dogs, enzyme histochemical studies of the rat papillae (19) show that glucose-6-phosphate dehydrogenase is active in the interstitial cells of this tissue. The main function of this cycle and its associated reactions is to supply NADPH to be used as the reducing agent in fatty acid synthesis. As already cited, the papillary interstitial cells in water-diuretic rats have a certain resemblance to leucocytes. Phagocytosis by guinea pig leucocytes is accompanied by a great increase in the ratio of $^{14}CO_2$ generated from [1-^{14}C]-glucose to $^{14}CO_2$ generated from [6-^{14}C]-glucose (from 8 to 22), indicating a specific stimulation of the pentose phosphate cycle (20). Since the interstitial cells probably are responsible for the major part of the papillary lipogenesis and the fatty acid synthesis is greatly inhibited when the tonicity of the media was changed by means of urea additions, it was tempting to see whether the high rate of lipid synthesis observed in media of 340 mosmol/kg H_2O compared to the low rate of synthesis in incubation media of high osmolality is caused by an activation of the shunt pathway.

However, such an assumption could not be confirmed. The CO_2 production from carbon-1 relative to glucose carbon-6 was determined in a few experiments. The ratio ± SD of $^{14}CO_2$ generated from [1-^{14}C]glucose to $^{14}CO_2$ from [6-^{14}C]glucose at 340 mosmol/kg H_2O was 2.0 ± 0.3 (n = 3) and at 1100 mosmol/kg H_2O 2.3 ± 0.6 (n = 4). This difference is not significant ($P > 0.1$) and the number of determinations is indeed very limited. It is quite clear though that there is no specific activation of the pentose phosphate cycle at low osmolality.

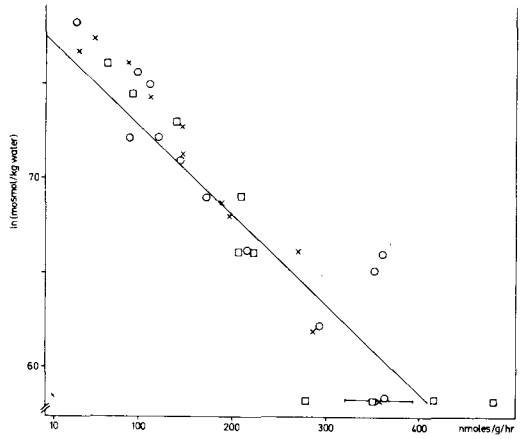

FIG. 2. Osmolality (urea addition) dependency of in vitro rate of [U-^{14}c]glucose incorporation into papillary lipids. Papillary slices randomly selected from 4 individual rats taken from 3 different groups were incubated 2 hr at 37 C with air as the gas phase in Krebs-Ringer phosphate buffer (340 mosmol/kg H_2O) containing 5 mM glucose and different amounts of urea in order to obtain different osmolalities. The line was drawn by the methods of least squares. Each value represents one incubation experiment. x = group I: rats with free access to water (normal rats); □ = group II: rats with only 1% NaCl-solution to drink for 48 hr (saline-loaded rats); ○ = group III: rats deprived of water for 24 hr (water-deprived rats).

DISCUSSION

In a previous study it was shown that in isosmotic media fatty acids exchange rapidly between the phospholipids and the triacylglycerols of the tissue (1,2). This exchange phenomenon was most pronounced in the locally synthesized 7,10,13,16-docosatetraenoic acid. Our attempt to investigate the effect of the addition of urea to the incubation media on the rate of this exchange of 7,10,13,16-docosatetraenoic acid failed since no change of sp act of the acid was observed as in isosmotic media during the incubation period. Without an asymmetry in the labeling of the 2 lipid classes, the exchange phenomenon can not be studied.

The lipogenesis from acetate in isosmotic media (in vitro) was qualitatively as well as quantitatively the same as in eviscerated rats (in vivo) (9). Recent studies showed that the incorporation of glucose into the glycerol moiety of papillary glycerolipids in vivo also is approximately the same as in vitro when the osmolality of the medium is isosmotic (10).

Since the osmolality of the papillary interstitium in normal rats is highly hypertonic these results seemed somewhat surprizing after the discovery of the importance of medium osmolality for papillary lipogenesis. However, the problem disappeared when it was found that the osmolality of the papillary interstitial fluids from eviscerated rats was as low as 463 ± 47 mosmol/kg H_2O (SD; n = 4) (Table I) a value at which the lipogenesis is not significantly lower than that in 340 mosmol/kg H_2O media (Fig. 2).

The effect of adding either urea or NaCl to the media on the glucose metabolism of papil-

lary slices from dogs have been studied by Bernanke and Epstein (17). They found a gradual depression of lactate production from glucose by increasing the osmolality of the medium to 1200 mosmol/kg H_2O. Hypertonicity appeared to exert a similar inhibition on oxygen uptake. In similar studies, Kean et al. (18) found a decreased rate of glycolysis by increasing the osmolality above 1300 mosmol/kg H_2O by adding NaCl. Above this level, the CO_2 formation and the rate of oxygen consumption were also inhibited. In agreement with these data, it was noticed during our studies that the $^{14}CO_2$ formation from [^{14}C]-glucose and the oxygen uptake were depressed in parallel with the glycerolipid formation as the osmolality of the media was increased by urea addition (data not shown).

A significant proportion of glucose metabolized by the renal papillae is processed through the pentose phosphate cycle. In leucocytes, high osmolality has been shown to inhibit specifically the oxidation of [1-^{14}C]glucose to $^{14}CO_2$ (8); therefore, the effect of adding urea to the incubation media on this pathway was studied. However, no specific effect on the CO_2 production from carbon-1 of glucose via the phosphogluconate oxidative pathway was seen. On the basis of this observation and of the effect of osmolality on the energy metabolism, it is most likely that the effect of urea on papillary lipogenesis is caused by a general metabolic inhibition in the tissue.

Danon et al. (21) have studied the papillary prostaglandin biosynthesis in hypertonic buffers after the addition of NaCl, sucrose and urea. Independent studies in our laboratory (unpublished data) have confirmed the effect of urea on the rate of PGE_2 biosynthesis. In contrast to the effect on the synthesis of glycerolipids, the rate of prostaglandin production was enhanced by increasing osmolality to the same extent that the lipid synthesis was abrogated when the effect of physiological concentrations of urea was investigated. It is therefore predicted that a shortage of glucose would enhance prostaglandin synthesis through decreased trapping of the prostaglandin precursor, free arachidonic acid. According to Tannenbaum et al. (22), this is true in fact, since they have found a high prostaglandin production when the glucose concentration is very low (less than 2 mM). This study therefore emphasizes that factors controlling lipogenesis determine the rate of prostaglandin synthesis just as well as the factors controlling lipolysis. Increased urea concentration in the renal papilla is an example of an increased prostaglandin synthesis as a consequence of decreased lipogenesis.

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Surface Viscosities of Phospholipids Alone and with Cholesterol in Monolayers at the Air-Water Interface

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ABSTRACT

Surface viscosities in lipid monolayers at the air-water interface were measured by the oscillating pendulum method. The logarithms of successive oscillations decreased linearly with time. Surface viscosity is reported here in terms of the rate constant, k, for decay of oscillation. Viscosities were measured as a function of surface pressure at 22 ± 2 C. Lipids investigated included cholesterol, 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PC), 4 other unsaturated PC, 1-palmitoyl-2-stearoyl PC, 1,2-distearoyl PC, 1-palmitoyl-lysophosphatidylcholine, 1-palmitoyl-lysophosphatidylserine, tetrapalmitoyl bisphosphatidic acid, and the dipalmitoyl species of PC, phosphatidylethanolamine (PE), phosphatidyldimethylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidic acid. Pressure-area curves are presented for the saturated phospholipids. Surface viscosities of most of the phospholipids were high and increased with increasing surface pressure. However, surface viscosities in monolayers of cholesterol, 1-palmitoyl-2-arachidonoyl PC, lysophosphatidylcholine or lysophosphatidylserine were very low and barely detectable under our experimental conditions. One mol % of cholesterol in monolayers of dipalmitoyl PC greatly reduced the surface viscosity of the film and, in mixed films containing 10% or more of cholesterol, surface viscosity was too low to measure. Cholesterol also reduced surface viscosities in monolayers of the other dipalmitoyl phospholipids. It is suggested that cholesterol functions in lung surfactant by reducing the surface viscosity of its highly saturated phospholipid components.

INTRODUCTION

The monomolecular film at the air-water interface is the simplest possible model for studying the properties of the amphiphilic molecules, including sterols and phospholipids, that are vital parts of the biological membrane. Advantages of the monolayer system include simplicity of geometry (no curvature), control of surface pressure and molecular area, absence of potentially misleading probe molecules and economy in materials and equipment. The lipid monolayer at the air-water interface is also a useful model for the study of lung surfactant (see Discussion).

The mobilities of lipid molecules in biological membranes are very important for their functions in these membranes. Numerous investigations of the fluidity of lipids in bilayer membrane models have been made by various techniques, including nuclear magnetic resonance (NMR), electron spin resonance (ESR), and fluorescence depolarization, but there are relatively few studies of viscosities in monolayer films. It seemed valuable, therefore, to measure surface viscosities of various different lipid structures in the relatively simple monolayer system.

The 3 techniques by which surface viscosities are most often measured are the canal or slit viscometer (suitable for very low viscosities), the rotating ring viscometer and the oscillating pendulum method. Theories and equations for interpretation of observations made by these methods are given by Gaines (1), Adamson (2), Joly (3) and Tschoegl (4; oscillating pendulum only). The different methods differ in sensitivity and results obtained by one method are usually not in quantitative agreement with those obtained by another.

An extraordinarily high surface viscosity has been observed in dipalmitoyl phosphatidylethanolamine (PE) and other saturated phospholipids, as measured by the oscillating pendulum method (5-7). We therefore used this method to study surface viscosities in a series of different phospholipids and to investigate the effects of cholesterol on these surface viscosities. Surface viscosities of most of the fully saturated phospholipids were very high, but viscosities of unsaturated phospholipids were too low to measure under our conditions. The high surface viscosities of saturated phospholipids were profoundly reduced by 1 or 2 mol % of cholesterol.

MATERIALS AND METHODS

Pressure-Area Measurements

Measurements were made at 22 ± 2 C with a surface balance (Cenco Hydrophil Balance, Central Scientific Co., Chicago, IL) as described earlier (8,9). The temperature for one set of measurements run on the same day did not vary more than ± 0.5 C. A lipid solution in 50 μl of benzene/chloroform/methanol (90:10:6, by vol) was applied onto the water surface (glass-

distilled, pH ca. 5.2) with a Hamilton syringe and the measurements, started 2 min later, took ca. 10 min. Two or more films of each composition were measured and duplicate aliquots of every sample were removed at time of spreading for measurement of fatty acid content by gas liquid chromatography (GLC).

Surface Viscosity Measurements

Surface viscosity was measured by the oscillating pendulum method (1-4). A cylindrical pendulum suspended by a thin wire was caused to oscillate on either a clean water surface or on a lipid film maintained at a known surface pressure (2, 5, 10, 15, 20, 30, 40, 50 or 60 dynes/cm). The rate of damping of oscillations was measured.

The pendulum bob was a cylinder of brass and teflon, 3 cm diameter, weight 238 g and moment of inertia 658 g cm^2. The tungsten suspension wire, ca. 0.012 cm in diameter, had a torsion constant of 131 dyne cm. The pendulum assembly was lowered so that the teflon base just made contact with the surface. The period of oscillation of this assembly was 14.1 sec. Amplitudes of oscillation were measured by reflecting a light beam (Model 155 He-Ne laser, 0.95 mw, 623 nm; Spectra Physics Inc., Mountain View, CA) from a mirror on the suspension wire onto a graduated scale. The number of swings observed with each film under each condition ranged from 3 to over 100. The logarithms of the amplitudes plotted vs swing number produced straight or nearly straight lines (Fig. 1). From plots of amplitude vs time, the rate of decay of oscillation, k sec^{-1}, was obtained. On a clean water surface, the value of k was ca. 9 x 10^{-4} sec^{-1}. This value was subtracted from values of k obtained with monolayers to provide a measure of the surface viscosity of the monolayer. On a given day, duplicate measurements of k agreed within a factor of 2 or less, but variation from day to day was greater, possibly because of differences in temperature. Despite this variation, values obtained for different films or at different pressures showed little overlap. k Values ranged from k = 0.1 x 10^{-4} to 3600 x 10^{-4} sec^{-1}. Standard errors of the mean values of k are shown in the figures and tables.

Lipids

Cholesterol and some of the phospholipids were purchased from commercial sources (Table I). Mixed acid phosphatidylcholines (PC) were synthesized by the anhydride method and purified as described earlier (8). Each phospholipid migrated as one component during thin layer chromatography (TLC) (10).

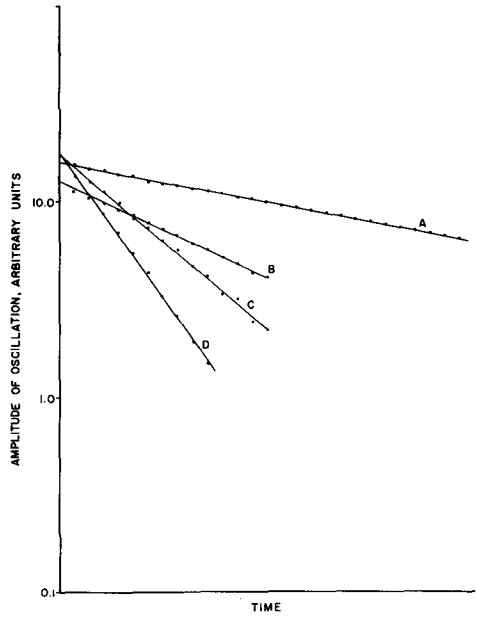

FIG. 1. Damping of oscillations by dipalmitoyl phosphatidylcholine monolayers at different surface pressures. Logarithms of amplitude in arbitrary units is plotted vs time (swings). The interval between points is 7.05 sec. A: 30 dynes/cm; B: 40 dynes/cm; C: 50 dynes/cm; and D: 60 dynes/cm. Subphase, double-distilled water, pH ca. 5.2.

Fatty acids in each phospholipid were converted to methyl esters and analyzed quantitatively by GLC as described earlier (8). The purity of cholesterol was measured by GLC of the free sterol (8). Purities of the lipids are given in Table I.

In addition to the compounds listed in Table I, the surface viscosities of the following PC: palmitoyl-arachidonoyl, palmitoyl-oleoyl, palmitoyl-α-linolenoyl, palmitoyl-γ-linolenoyl, and palmitoyl-linoleoyl were also studied. Viscosities of these films were indistinguishable from that of a clean water surface as was the viscosity of cholesterol and of mixed films containing cholesterol and palmitoyl-arachidonoyl PC (2, 5, 10, 20, 30, 50 mol % cholesterol at 40 dynes/cm). In contrast, viscosities of some of the saturated phospholipid films were so high that measurement was impossible because the pendulum was immobilized in less than 3 swings.

RESULTS

Pressure-Area Measurements

Molecular areas of PC decreased in the order 16:0-16:0 > 16:0-18:0 > 18:0-18:0 (Fig. 2A). The molecular area of dipalmitoyl PE was

smaller than those of the PC (Figs. 2B and 2A). In the series dipalmitoyl PE, dipalmitoyl phosphatidyl N-methylethanolamine, dipalmitoyl N,N-dimethylethanolamine and dipalmitoyl PC, each additional methyl group increased the molecular area in the film (Fig. 2B). All saturated PC and PE monolayers could withstand surface pressures of 60 dynes/cm or more.

Pressure-area curves for phospholipids with 1, 2 or 4 palmitoyl chains per molecule are shown in Fig. 2C. The curves for phosphatidylglycerol and phosphatidic acid are similar to each other and to the curve of distearoyl PC (Fig. 2A). Tetrapalmitoyl bisphosphatidic acid occupied roughly twice the molecular area shown by dipalmitoyl phospholipids. Films of these 3 phospholipids could be compressed to pressures above 60 dynes/cm, but 1-palmitoyl-lysophosphatidylcholine and 1-palmitoyl-lysophosphatidylserine could not be compressed above ca. 30 and 40 dynes/cm, respectively. The 1-palmitoyl-lysophosphatidylcholine and 1-palmitoyl-lysophosphatidylserine also formed widely expanded films. The unsaturated PC formed relatively expanded films and could not be compressed above ca. 45-50 dynes/cm (data not shown). The pressure-area curve of stearic acid, measured earlier in this laboratory under very similar conditions (11), is included here to show the molecular area of a single saturated chain.

Some of the pressure-area data are replotted in Fig. 2D to show the areas per acyl chain in molecules with 1, 2 or 4 saturated chains per molecule. These curves indicate that the structure of the polar group has an enormous effect on the molecular areas and compressibilities of the films.

TABLE I

Sources and Purities of Lipids Used in Surface Viscosity Measurements

Name	Abbreviation	Purity of fatty acid or sterol	Source
Dipalmitoyl phosphatidylcholine	di 16:0 PC	> 99%	Sigma Chem. Co., St. Louis, MO
1-Palmitoyl-lysophosphatidylcholine	LPC	> 99%	Sigma Chem. Co., St. Louis, MO
Dipalmitoyl phosphatidic acid	di 16:0 PA	> 99%	Sigma Chem. Co., St. Louis, MO
Dipalmitoyl phosphatidylglycerol	di 16:0 PG	> 99%	Serdary Research Labs., London, Ontario
Dipalmitoyl-N-methyl phosphatidylethanolamine	di 16:0 PMME	> 99%	Calbiochem, La Jolla, CA
Dipalmitoyl-N,N-dimethylphosphatidylethanolamine	di 16:0 PDME	> 99%	Sigma Chem. Co., St. Louis, MO
Dipalmitoyl phosphatidylethanolamine	di 16:0 PE	> 99%	Calbiochem, La Jolla, CA
Distearoyl phosphatidylcholine	di 18:0 PC	> 99%	Calbiochem, La Jolla, CA
1-Palmitoyl-2-stearoyl phosphatidylcholine	16:0-18:0 PC	49.9% 16:0, 50.1% 18:0	(synthesized)
Tetrapalmitoyl bisphosphatidic acid	16:0 BPA	> 99%	Serdary Research Labs., London, Ontario
1-Palmitoyl-lysophosphatidylserine	LPS	> 99%	Serdary Research Labs., London, Ontario
Cholesterol		> 99%	Nutritional Biochem. Co., Irvine, CA

Surface Viscosities

Surface viscosities, expressed as rate constants for decay of oscillation, k sec^{-1}, are shown in Figure 3A-C. Surface viscosities of PC increased with increasing surface pressure and increasing chain length and became immeasurably high (the pendulum was immobilized) above ca. 40 and 30 dynes/cm for palmitoyl-stearoyl PC and distearoyl PC, respectively (Fig. 3A).

Surface viscosities of dipalmitoyl PE and its N-methylated derivatives decreased as the degree of methylation increased (Fig. 3B). The surface viscosity of unsubstituted dipalmitoyl PE became unmeasurably high above 10 dynes/cm.

Surface viscosities of phosphatidic acid, phosphatidylglycerol and bisphosphatidic acid (all palmitoyl derivatives) behaved similarly

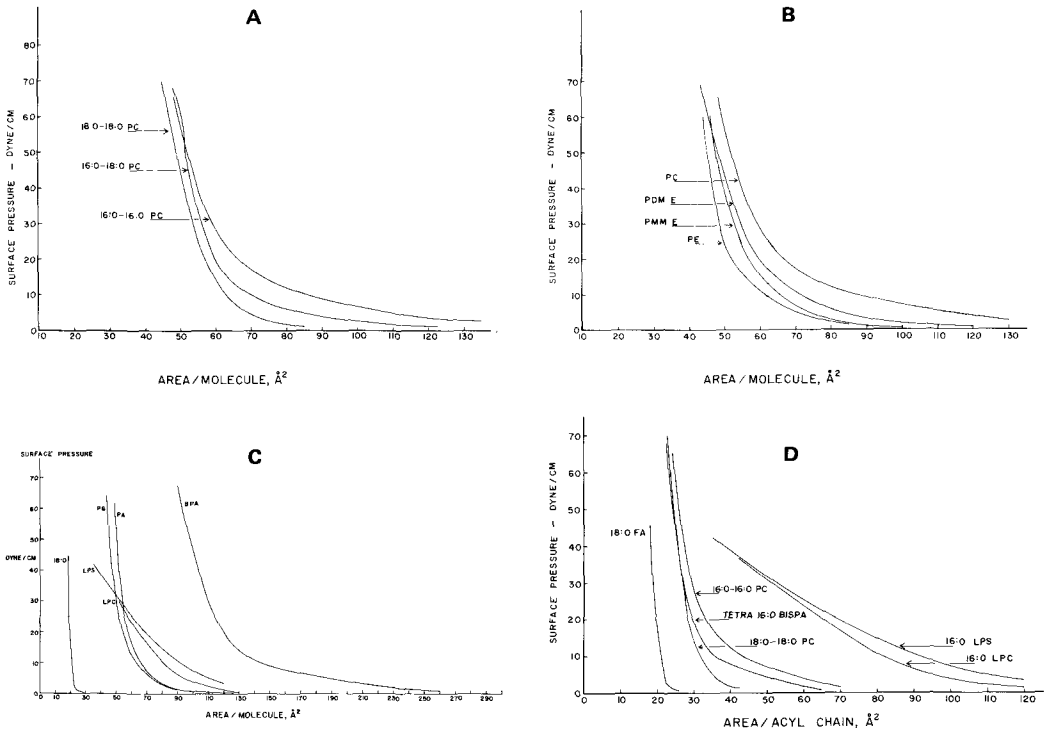

FIG. 2. (A) Pressure-area curves of saturated phosphatidylcholines at the air-water interface at 22 ± 2 C. The subphase was glass-distilled water, pH ca. 5.2; (B) pressure-area curves of dipalmitoyl phosphatidylethanolamine and its N-methylated homologs at the air-water interface at 22 ± 2 C. The subphase was glass-distilled water, pH ca. 5.2; (C) pressure-area curves of phospholipids with 1, 2 or 4 palmitoyl chains/molecule at the air-water interface at 22 ± 2 C. The subphase was glass-distilled water, pH ca. 5.2; (D) pressure-area curves at the air-water interface at 22 ± 2 C for various saturated lipids plotted to show area/saturated acyl chain. The subphase was glass-distilled water, pH ca. 5.2.

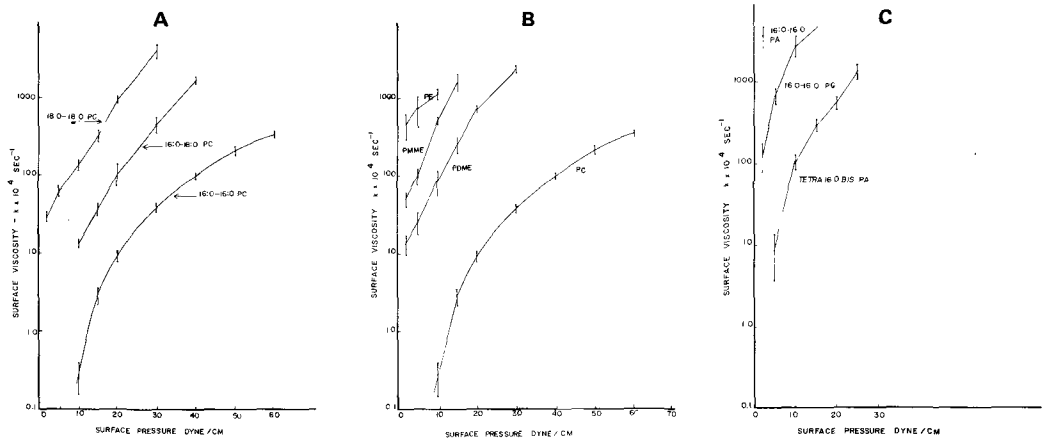

FIG. 3. (A) Surface viscosities of saturated phosphatidylcholines as a function of surface pressure. Subphase was glass-distilled water, pH ca. 5.2 and the temperature was 22 ± 2 C; (B) surface viscosities of dipalmitoyl phosphatidylethanolamines and its N-methylated homologs as a function of surface pressure. Subphase was glass-distilled water, pH ca. 5.2; (C) surface viscosities as a function of surface pressure for the palmitoyl analogs of phosphatidic acid, phosphatidyl glycerol and bisphosphatidic acid. Subphase was glass-distilled water, pH ca. 5.2.

to the other phospholipids in that viscosity increased with increasing surface pressure (Fig. 3C). The viscosity of dipalmitoyl phosphatidic acid was too high to measure above a pressure of 2 dynes/cm. Surface viscosities of lysophosphatidylcholine and lysophosphatidylserine were too low to measure between 2 dynes/cm and 30 dynes/cm.

The effects of cholesterol on surface viscosities in films of dipalmitoyl PC are shown in Figure 4A and Table II. One mol % of cholesterol reduced the surface viscosity of the mixed film by a factor of 6. At concentrations of cholesterol above 15 mol % surface viscosity became immeasurably low.

Cholesterol also reduced the surface viscosities of dipalmitoyl phosphatidic acid, dipalmitoyl phosphatidylglycerol, tetrapalmitoyl bisphosphatidic acid and dipalmitoyl PE and its methylated derivatives (Table III). These measurements were made at surface pressures just below that at which the viscosities of the pure films became too great to measure, except for dipalmitoyl PC-cholesterol mixtures, which were measured at 40 dynes/cm.

DISCUSSION

Pressure-Area Curves

Molecular areas decreased with increasing chain length in the saturated PC molecules (Fig. 2A). This phenomenon has been reported before in homologous series of saturated phospholipids (12,13). The decrease in molecular area with increasing chain length can be attributed to London-Van der Waals attractive forces, the strength of which increases with the number of methylene units in a saturated chain and increases as the chains become closer together (14).

The presence of a glycerylphosphoryl group greatly increased molecular area, as can be seen by comparison of the areas/acyl chain in phospholipid monolayers with that of stearic acid (Fig. 2D). The molecular area of dipalmitoyl PC was much greater than that of dipalmitoyl PE, an effect which may be caused by hydration of the choline or phosphorylcholine group. In phospholipid bilayers, hydration of the phosphorylcholine group is much greater than that of the phosphorylethanolamine group (15,16). The intermediate molecular areas of partially methylated PE may indicate intermediate levels of hydration.

Surface Viscosity

Surface viscosity of all films increased with increasing surface pressure (Figs. 3A-C). When the molecules are closer together, the attractive

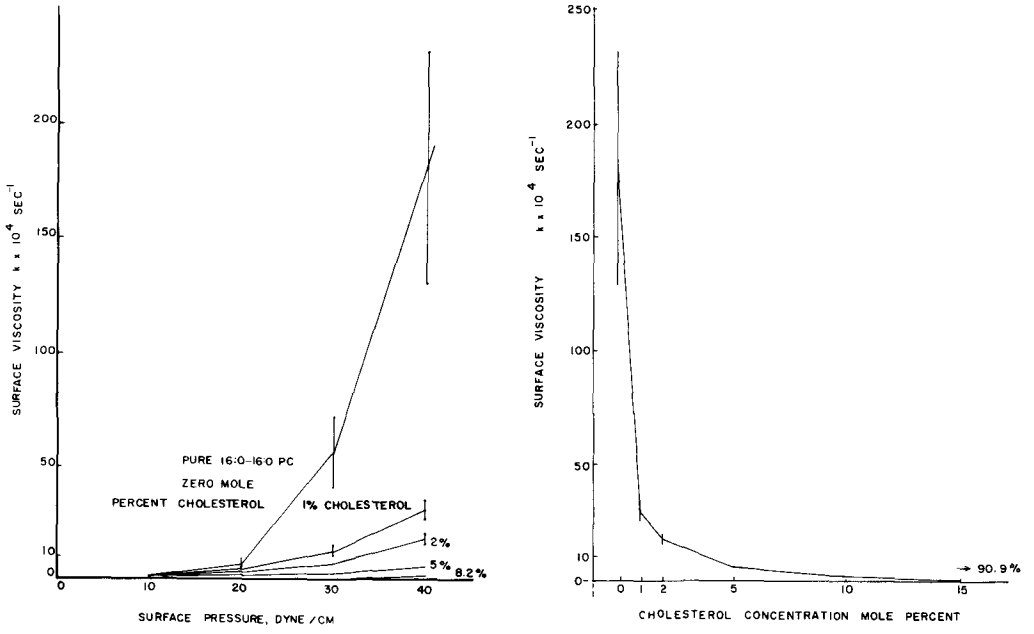

FIG. 4. (A) Effect of cholesterol on surface viscosity of dipalmitoyl phosphatidylcholine as a function of surface pressure and cholesterol concentration. Subphase was glass-distilled water, pH ca. 5.2; (B) Surface viscosity in monolayers of dipalmitoyl phosphatidylcholine at the air-water interface as a function of cholesterol concentration. Surface pressure = 40 dynes/cm. Subphase was glass-distilled water, pH ca. 5.2.

TABLE II

Surface Viscosities in Mixed Monolayers of Cholesterol and Dipalmitoyl Phosphatidylcholine

Film composition		Surface viscosity ($k \times 10^4$)[a]			
		Surface pressure (dynes/cm)			
Mol % cholesterol	Phospholipid:sterol mol ratio	10	20	30	40
None; pure phospholipid	∞	0.9 ± 0.7	5.4 ± 2.8	55 ± 15.6	180 ± 51
1	99:1	0.5 ± 0.1	4.0 ± 0.9	11.8 ± 2.0	30.0 ± 3.8
2	49:1	NM[b]	1.7 ± 0.4	6.9 ± 0.6	17.6 ± 1.9
5	19:1	NM	1.3 ± 0.04	2.7 ± 0.2	5.4 ± 0.06
9.2	9.9:1	NM	NM	0.8 ± 0.7	1.5 ± 0.4

[a]Surface viscosities expressed as $k \times 10^4$ sec^{-1}, where k is rate of decay of oscillation. Values are means ± SEM.

[b]NM, not measurable.

forces between acyl chains are greater, and this should increase the surface viscosities as pressure increases. If attractive forces between hydrocarbon chains were the sole source of surface viscosity, then the surface viscosities of all the dipalmitoyl phospholipids should be the same at the same molecular areas, regardless of the structures of the polar groups. However, the surface viscosity of dipalmitoyl phosphatidic acid was extremely high at a molecular area of 85 $Å^2$, whereas that of dipalmitoyl PC was too low to measure at this molecular area (Fig. 5). This indicates that interactions between polar groups contribute to high values of surface viscosity.

Effects of Cholesterol on Surface Viscosities

Cholesterol, which has a relatively low surface viscosity, profoundly reduced the surface viscosities of saturated phospholipids in monolayers (Figs. 4A-B; Tables II and III). It is notable that only 1 mol % of cholesterol in a film of dipalmitoyl PC reduced the surface viscosity by a factor of 6. Two mol % of cholesterol also greatly reduced surface viscosities in monolayers of other palmitoyl phospholipids, except for dipalmitoyl phosphatidic acid (Table III). These great reductions in surface viscosity imply that 1 cholesterol molecule can influence the behavior of a large number of phospholipid molecules. Cholesterol can interact either with the polar groups or with the acyl chains of phospholipids, as has been shown by spin label studies (17), NMR investigations (18,19), Raman spectra (20) and fluorescence polarization and quenching measurements (21). The possibility that strong interactions between polar groups are necessary for high surface viscosities suggests that cholesterol is interfering with these strong polar group interactions.

High surface viscosities have been attributed to a polymeric structure or aggregation of the molecules in monolayers of dipalmitoyl PE and other saturated phospholipids. Hayashi and colleagues (5,6,22,23) and Standish and Pethica (24) have suggested a 2-dimensional crosslinked lattice model in which molecules of PE are held together by attractive forces between unlike charges on the polar groups.

A 1-dimensional linear polymeric model could also account for high surface viscosities. Let us assume (a) that saturated phospholipid molecules, in particular dipalmitoyl PC, can bind or associate firmly with other PC molecules in the monolayer film to form long strings or chains of molecules, strings that can slip by each other. Because the forces between molecules are weak, the aggregates may be

TABLE III

Relative Surface Viscosities of Mixed Monolayers Containing Cholesterol and Palmitoyl Phospholipids

Phospholipid	Viscosity of mixed film / Viscosity of pure phospholipid film: Cholesterol concentration (mol %) 2	20	Surface pressure (dynes/cm)
16:0-16:0 PC	0.093	0	40[a]
16:0-16:0 PDME	0.058	0.001	30
16:0-16:0 PMME	0.224	0.003	15
16:0-16:0 PE	0.674	0.020	5
16:0-16:0 PA	1.010	0.002	2
Tetra-16:0 BPA	0.363	0.085	30
16:0-16:0 PG	0.010	0.010	5

[a]The viscosity of a mixed film, except for PC, was measured at a surface pressure slightly below that at which the viscosity of the pure phospholipid film became unmeasurably high. The pressure at which viscosity became unmeasurably high was above 60 dynes/cm for PC.

continually breaking and reforming, so that the size (chain length) of the aggregate will be an average that depends on surface pressure, temperature and other conditions.

Let us also assume (b) that cholesterol can bind strongly to 1 and only 1 dipalmitoyl PC molecule in a monolayer. This would result in chains of PC molecules with a cholesterol molecule bound tightly at 1 or both ends of the chain. If the concentration of cholesterol were high, PC chain lengths would be short, and at low concentrations of cholesterol, chain lengths would be long. At very low concentrations of cholesterol, there would be too few cholesterol molecules to occupy all the chain ends. At this concentration, cholesterol would have a negligible effect on the surface viscosity of the film.

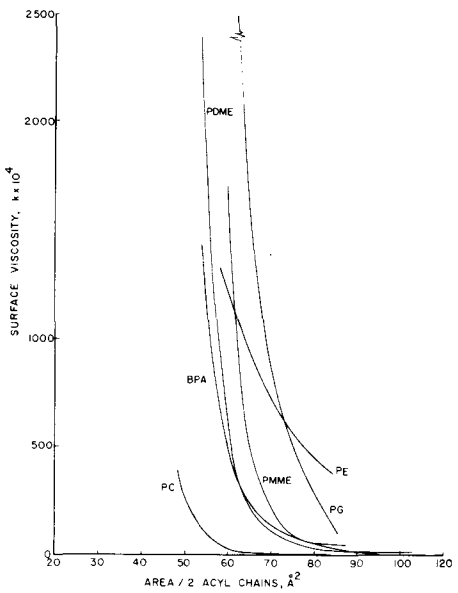

FIG. 5. Surface viscosity as a function of area/acyl chain in monolayers of saturated phospholipid monolayers. Subphase was glass-distilled water, pH ca. 5.2.

The viscosity of linear high polymers in solution is proportional to the molecular weight (MW) or chain length of the polymer (25):

$$\eta = (\text{constant})\, M^a,$$

where η = intrinsic viscosity of polymer in solution,

M = average MW of polymer, and

a = 1 for random chain; for a spherical particle, $a < 1$.

If surface aggregates behave analogously to 1-dimensional linear polymers, then surface viscosities should be directly proportional to the size of the surface aggregate or the chain lengths of the surface polymers. This appears to be true for mixtures of cholesterol and dipalmitoyl PC (Table II; Fig. 6). Surface viscosities were directly proportional to the molar ratio of PC-to-cholesterol. If the assumptions just given are true, we can find the size of the aggregates (chain lengths of the polymers) by extrapolating the surface viscosity vs molar ratio plot of mixed films to the k value observed in a film of pure PC. This gives the molar ratio of phospholipid-to-sterol at which surface viscosity is not affected by the presence of sterol. At this concentration, the number of sterol molecules is equal to the number of ends of PC chains available to be bound, so that binding of sterol does not change the chain lengths of the PC polymer. At 40 dynes/cm, we find a chain length of ca. 580 PC units, at 30 dynes the length is 450 PC units and at

20 dynes/cm the chain length is ca. 130 units (extrapolation not shown). These chain lengths are based on the assumption that cholesterol molecules can bind to only 1 end of a PC chain polymer. If cholesterol can bind at both ends, the values just given should be multiplied by 2.

Albrecht and colleagues have calculated the sizes of aggregates in monolayer films of dipalmitoyl PC undergoing phase transitions at temperatures ranging from below 10 C to 39 C or higher, at different pressures (27). Their estimates of the number of molecules per cooperative unit ranged from 60 to 190, depending on temperature and pressure. Their values and ours should not be compared directly because their combinations of temperature and pressure do not coincide with ours and because their data were calculated for monolayer films undergoing phase transitions. Nevertheless, their data and ours both suggest that dipalmitoyl PC films form aggregates that contain of the order of hundreds of molecules per unit.

This linear polymeric model could be tested by comparing the effects of different sterols on the surface viscosity of dipalmitoyl PC films. Different sterols may bind with different degrees of affinity, but the extrapolation of k values of mixtures to the k value of the pure film should give the same value for the chain length of the polymers in the pure PC film.

Physiological Significance

Lipid monolayers at the air-water interface may occur in vivo on the surfaces of the air passages in the lung. Lung alveoli in mammals are covered with a surfactant capable of lowering surface tension almost to zero (28). Surface tension is related to surface pressure by the equation:

$$\pi = \gamma_0 - \gamma,$$

where π = surface pressure, dynes/cm,
γ_0 = surface tension of subphase, dynes/cm,
γ = surface tension of film, dynes/cm.

Thus, a surface tension of zero corresponds to a surface pressure of ca. 72 dynes/cm at 20 C and ca. 70 dynes/cm at 37 C, the surface tensions of water at these temperatures. The low surface tension of surfactant prevents collapse of lung alveoli at low lung volumes. Lung surfactant contains considerable lipid, of which ca. 80% is phospholipid and the rest is cholesterol, triglyceride and other neutral lipid (29). Phospholipid of lung surfactant is unique in that it consists largely of PC (75-85% of total phospholipid) and very small proportions of PE, phosphatidyldimethylethanolamines, phosphatidylglycerol, sphingomyelin and others (29-31). Surfactant phospholipids contain unusually high proportions of saturated acyl chains, mainly palmitic (29-31). For example, alveolar PC from lambs amounted to 86% of total phospholipid and palmitic acid accounted for 82% of its total fatty acids (30). Unesterified cholesterol accounts for 52% (32) to 67% (29) of the neutral lipid in lung surfactant, so that the concentration of cholesterol in lung surfactant lipids is at least 10 mol %.

The formation of functional lung surfactant begins shortly before birth in animals (30,33) and human beings (31). Lipid analysis of lung washings from fetal animals and of amniotic fluid at various stages of human gestation reveal 2 important changes in lipid composition during maturation of lung function. First, the proportion of PC rises from the immature value of 40% to the mature level of 86% of total lipid phosphorus (30), and second, the degree of saturation of all the phospholipids increases to the high values observed in mature surfactant (30,31). These changes are accompanied by an increase in the ability of lung surfactant to reduce surface tension to very low values. It is probable that these very low surface tensions result from the presence of the highly saturated phospholipids (34), monolayers of which are able to attain very high surface pressures even when they contain 20-30 mol % of cholesterol.

If lung surfactant consisted of phospholipid alone, its surface viscosity would increase during maturation of lung function because of the increase in degree of saturation in the acyl chains. However, it has been observed that the

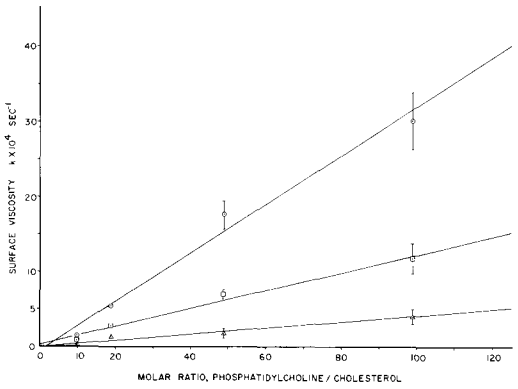

FIG. 6. Surface viscosities of mixed films of dipalmitoyl phosphatidylcholine and cholesterol as a function of composition and surface pressure. Circles, squares and triangles represent measurements at 40, 30 and 20 dynes/cm, respectively. Slopes and intercepts were calcd by the method of least squares (26). Correlations were 0.95, 0.93 and 0.85 for data at 40, 30 and 20 dynes/cm, respectively.

microviscosity measured by fluorescence polarization of lipids in amniotic fluid actually decreases as gestation progresses in humans. In fact, the reduction in viscosity was correlated with lung maturity as monitored by measurements of the PC:sphingomyelin ratio (35). This decrease in viscosity could result from an increase in the cholesterol content of the surfactant. Ness and colleagues (33) have suggested that in the rat, the formation of cholesterol in lung surfactant may increase 3 or 4 days before birth, because there is an increase in the activity of 3-hydroxy-3-methyl glutaryl CoA reductase in lung microsomes at this time. This coincides with the onset of active surfactant production in the rat. However, in the adult rat, the cholesterol of lung surfactant is largely derived from plasma lipoproteins (32). The maturation of lung surfactant therefore seems to include the following steps: (a) the proportion of PC increases to a very high percentage, 75% or more, of total phospholipid; it may be significant that dipalmitoyl PC exhibit lower surface viscosities than do dipalmitoyl phosphatidic acid and phosphatidylglycerol, which also occur in surfactant; (b) the degree of saturation in phospholipids increases, which enables monolayers of these molecules to attain very low surface tensions (high surface pressures); and (c) the microviscosity of the lipids in amniotic fluid decreases, possibly because of an increase in cholesterol concentration.

It is notable that a low viscosity would be produced if the lung surfactant phospholipids remained unsaturated, but then a very low surface tension would not be attainable.

These observations suggest that both the surface tension and the surface viscosity are critical for the normal functioning of lung surfactant. One might speculate that a low surface viscosity may facilitate the reformation of the monolayer film as it is repeatedly compressed and expanded in the lung alveoli during respiration. This possibility is supported by the observation that the addition of cholesterol to monomolecular films of dipalmitoyl PC improved the respreading properties of monolayer films upon dynamic compression past collapse (36).

The oscillating pendulum method for measuring surface viscosity is capable of measuring the effects of very small amounts of cholesterol in monolayers of the saturated phospholipid molecules reported here. Most of the other methods of measuring cholesterol-phospholipid interactions require the presence of 10 mol % or more of cholesterol to demonstrate an interaction.

ACKNOWLEDGMENTS

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Lipids of Myocardial Membranes: Susceptibility of a Fraction Enriched in Sarcolemma to Hydrolysis by an Exogenous Mammalian Phospholipase A_2[1]

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ABSTRACT

A myocardial membrane fraction enriched in sarcolemma was used to determine the susceptibility of the lipids to hydrolysis by a phospholipase A_2 from granulocytes. After incubation (37 C, pH 7.0, 5 mM Ca^{2+}) with the phospholipase A_2 for 30 min, a more than 3-fold increase in unesterified fatty acids was found (up to 47 nmol/mg protein; $P < 0.001$) relative to a control incubated without phospholipase A_2 or Ca^{2+}. This included a 10-fold increase in the arachidonic acid content (up to 42 mol %) and at least a 7-fold increase in lysophosphatidylethanolamine (up to 7.4 mol % total phospholipid-P). However, the exogenous phospholipase did not augment the quantity of lysophosphatidylcholine produced by endogenous phospholipases in the presence of Ca^{2+} (5 mM). These results demonstrate the in vitro susceptibility of phospholipids of myocardial membranes, particularly phosphatidylethanolamine, to the neutral-active, Ca^{2+}-dependent phospholipase A_2 from granulocytes. This work may be relevant to myocardial inflammation and tissue damage during ischemia, where heterolytic injury of the myocardium may occur subsequent to the accumulation of granulocytes.

INTRODUCTION

Myocardial injury during acute inflammation and ischemia may involve hydrolysis of lipids of subcellular membranes by phospholipases (PLA) which have been found in cardiac tissue (1-4). The process of injury during ischemia includes increased permeability of the sarcolemma (5,6), alterations in the morphology of other subcellular structures (7) and reduction of latency of lysosomal hydrolases (8). During ischemia, significant increases have been found in unesterified long chain fatty acids in whole myocardial tissue (9) and in lysosome-rich fractions from cardiac tissue (10). Increased quantities of lysophospholipids in whole myocardial tissue have been found (11). We have reported previously (12) that enriched preparations of cardiac sarcolemma contain Ca^{2+}-stimulated PLA which hydrolyzes exogenous 2-[^{14}C]phosphatidylethanolamine optimally at neutral pH. More recently we have shown similar hydrolysis of endogenous phosphatidylcholine (PC) of a membrane preparation enriched in sarcolemma (13). In this work, we have studied the susceptibility of lipids of this myocardial membrane fraction to hydrolysis by a soluble PLA_2 from granulocytes (14).

MATERIALS AND METHODS

Preparation of Membrane Fraction

Dogs were anesthetized by intravenous administration of pentobarbital (27 mg/kg). Ventilation was maintained using an endotracheal tube attached to a Harvard respirator. Following rapid excision, the heart was cooled in ice-cold saline. The isolation of the sarcolemmal fraction by sucrose-gradient centrifugation has been described previously (15). This fraction was found to band at a sucrose concentration of 48-52% (w/w). It showed a 7-fold increase over the homogenate in the activity of ouabain-sensitive, (Na^+ + K^+)-stimulated, Mg^{2+}-ATPase (E.C. 3.6.1.3). It was diluted 3-fold with ice-cold imidazole buffer (20 mM, pH 7.0) and following centrifugation at 140,000 x g max for 30 min the pellet was resuspended gently in sucrose (0.25 M) buffered with imidazole (20 mM, pH 7.0).

PLA_2 from Granulocytes

The exogenous neutral-active, Ca^{2+}-dependent PLA_2 was obtained from rabbit granulocytes that were harvested from glycogen-induced peritoneal exudates (14). The specific activity (sp act) was assayed by hydrolysis of phospholipid of autoclaved *Escherichia coli* and was 669 nmol free fatty acid/mg protein/hr. The preparation showed no PLA_1 activity.

Incubation of the Membrane Fraction

Preliminary studies indicated that this membrane fraction hydrolyzed 1-acyl-2-[1-^{14}C]linoleoyl-*sn* glycero-3-phosphatidylethanolamine optimally at 37 C in the presence of 5 mM calcium at pH 7.0 (12). Thus, these conditions of incubation also were used for the

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examination of lipolysis of endogenous sarcolemmal lipids by the PLA_2. Sarcolemma (1 mg protein/ml) was incubated in a solution containing the exogenous PLA_2 (0.75 mg protein/ml), calcium chloride (5 mM) and imidazole-HCl buffer (20 mM, pH 7.0) for 30 min at 37 C in a shaking water bath. Control incubations with or without exogenous PLA_2 and Ca^{2+} added individually also were performed. The level of endogenous calcium in the sarcolemmal fraction was found to be 20 nmol/mg protein by atomic absorption spectroscopy. Membrane protein was assayed by the Lowry et al. method (16) with the addition of deoxycholic acid (0.3 ml of a 1% solution) using bovine serum albumin (BSA) as the standard.

Extraction and Analysis of Lipids

Incubations of the sarcolemmal fraction were terminated by the addition of cold methanol (6.3 times the aqueous sample vol). Heneicosanoic acid was added as the internal fatty acid standard. Lipids were extracted by the Folch et al. method (17) with minor modifications, including the determination of recovery factors for lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) as described previously (13). The stated quantities of lysophospholipids have been multiplied by these recovery factors (1.23 for LPC and 1.38 for LPE). Total phospholipid-P was determined (18). The composition of individual phospholipids of each sample was determined by 2-dimensional thin layer chromatography (TLC) (19); this technique was modified to include drying of the plates in vacuo for 30 min between developments. Mean recovery of the phosphorus content of the spots and equivalent blank areas was 97.3 ± 9.3 (SD) %. Total lipid extracts were separated on Silica Gel GHR (Mallinkrodt, Darmstadt) using redistilled petroleum ether (60-80 C)/diethylether/glacial acetic acid (80:20:1, by vol), containing butylated hydroxytoluene (0.1 mg/100 ml). Marker lipids were visualized using iodine vapor. Covered areas containing sample phospholipids, fatty acids, triacylglycerols, cholesteryl esters or blank areas were scraped into vials, sealed under N_2 and methylated at 100 C in boron trifluoridemethanol (15% w/v, Applied Science, Inc., State College, PA); the same vol of ice-cold sodium hydroxide (5 N) as BF_3-methanol reagent was added prior to extraction with pentane (20). The content of long chain methyl esters and dimethylacetals (derived from plasmalogens) was determined by gas liquid chromatography (GLC) with a glass column containing EGSS-X (10% w/w) on Gas Chrom P (Applied Science). Identities of the methyl esters and dimethylacetals were confirmed by mass spectrometry. Following the separation of phospholipids (19), the fatty acids and alk-1-enyl ethers were quantified; the solvent was removed from the plate in vacuo and the phospholipids were visualized in ultraviolet (UV) light using Rhodamine-6G (0.01% in methanol/water, 1:1, v/v, Eastman-Kodak, Rochester, NY). The spots were scraped into glass vials and the lipids were methylated as already described.

Differences between mean values (± SD) from 5 experiments were tested for statistical significance ($P < 0.05$) by the paired Student's t-test.

RESULTS

When the membrane fraction was incubated at 37 C without addition of PLA_2 or Ca^{2+} (Table I), the content of unesterified fatty acids was not significantly different in total quantity or distribution profile to that previously found in the unincubated preparation (13). Incubation with PLA_2 in the absence of added Ca^{2+} produced small but significant increases in the content of unsaturated fatty acids; these changes may have resulted from lipolysis under the stimulation of the PLA_2 and endogenous lipases by the low level of Ca^{2+} bound to the membrane (mean value 20 nmol/mg protein). Incubation performed in the presence of added Ca^{2+} alone doubled the total fatty acid content of the incubated control, the increase involving most species of fatty acids as described previously (13). Incubation with PLA_2 plus Ca^{2+} (5 mM) produced more than a 3-fold increase ($P < 0.001$) in total fatty acids (14.6 up to 47.2 nmol/mg protein). This represents the combined endogenous lipolysis and PLA_2-catalyzed hydrolysis of lipids. This included a 10-fold increase in unesterified arachidonic acid (2.0 up to 19.9 nmol/mg protein) which accounted for 49% of the total increase in fatty acids in the presence of PLA_2 plus Ca^{2+}. The increase ($P < 0.01$) in total fatty acids attributable to the Ca^{2+}-stimulation of the PLA_2 (16.1 nmol/mg protein) is calculated from the difference between incubations with PLA_2 plus Ca^{2+} and Ca^{2+} alone (Table I); this consisted almost entirely of an increase ($P < 0.001$) in arachidonic acid.

The quantity of total phospholipid-P did not change significantly from the incubated control level (457.4 ± 21.7 nmol/mg protein) as a result of incubation with PLA_2 alone (466.4 ± 18.1 nmol/mg protein) or with PLA_2 plus Ca^{2+} (462.6 ± 23.5 nmol/mg protein) indicating that

TABLE I

Content of Unesterified Fatty Acids of the Sarcolemma-enriched Fraction during Incubations to Enhance Lipolysis

Additions	None (control)	PLA_2	Ca^{2+}	$PLA_2 + Ca^{2+}$
Palmitic	3.6 ± 1.8[a]	4.2 ± 1.5	5.8 ± 2.8	4.1 ± 1.7
Palmitoleic	1.1 ± 0.6	1.0 ± 0.2	1.3 ± 0.6	0.9 ± 0.7
Stearic	2.0 ± 0.5	2.7 ± 0.8	4.3 ± 1.5[c]	2.7 ± 0.8
Oleic	3.9 ± 0.6	5.4 ± 0.9[b]	9.4 ± 2.7[c]	9.5 ± 0.6[d]
Linoleic	2.2 ± 0.4	4.2 ± 0.6[d]	6.5 ± 1.4[d]	9.4 ± 1.3[d]
Eicosatrienoic	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.5 ± 0.2[b]
Arachidonic	2.0 ± 0.2	3.7 ± 1.1[b]	3.4 ± 0.8[c]	19.9 ± 2.8[d]
Total	14.6 ± 3.1	21.3 ± 4.0[b]	31.1 ± 8.9[c]	47.2 ± 3.5[d]

The sarcolemmal fraction (1 mg protein/ml) was incubated at 37 C for 30 min at pH 7.0: with no addition, with PLA_2 (0.75 mg protein/ml); with calcium (5 nmol/ml); or with $PLA_2 + Ca^{2+}$.

[a]nmol/mg protein ± SD.

[b,c,d]Signify $P < 0.05$, 0.01, 0.001, respectively, for significant differences between incubated samples and incubated control. See Discussion concerning significance of differences between $PLA_2 + Ca^{2+}$ and Ca^{2+} incubations.

a significant production of water-soluble glycero-phosphate esters did not occur under these conditions.

Table II shows that incubation in the presence of PLA_2 plus Ca^{2+} increased significantly the content of LPE (up to 7.4 mol %) with a concomitant significant loss of phosphatidylethanolamine (PE); a limited formation of LPE occurred in the presence of PLA_2 without addition of Ca^{2+}. However, the level of LPC after incubation with PLA_2 plus Ca^{2+} was not significantly greater than the level of LPC in the presence of Ca^{2+} alone; LPC was not formed in the absence of added Ca^{2+}. The quantities of other phospholipids present in the unincubated sample described previously (13) were not altered significantly by any of the incubation conditions just described. Thus, only PE was subject to extensive attack by the exogenous Ca^{2+}-dependent PLA_2. Interestingly, the presence of PLA_2 appeared to inhibit the activity of triacylglycerol lipase since incubation in the presence of PLA_2 alone or PLA_2 plus Ca^{2+} was not associated with production of fatty acids from triacylglycerols (unincubated control: 41.2 ± 7.4, PLA_2 alone: 38.7 ± 4.9, $PLA_2 + CA^{2+}$: 40.4 ± 5.0 nmol of total fatty acid esterified as triacylglycerol/mg protein).

The esterified fatty acids and alk-1-enyl ethers of the parent PC, PE, and the corresponding lysophospholipids are shown in Table III. The LPC contained predominantly (89 mol %) palmitic acid, palmitoyl-1-enyl ether and stearic and oleic acids. LPE contained 83 mol % of stearic acid and stearoyl-1-enyl ether. However, the LPC and LPE also contained linoleic and arachidonic acids. The predominance of polyunsaturated fatty acids in the 2 *sn*-position in mammalian tissues (21) suggested that ca. 11 mol % of the LPC and 6 mol % of the LPE were acylated in the 2 *sn*-position. The chromatographic system could not distinguish between 1- and 2-acyl-lysophospholipids.

TABLE II

Composition of Choline and Ethanolamine-containing Phosphoglycerides of the Sarcolemma-enriched Fraction during Incubations to Enhance Lipolysis

Additions	None (control)	PLA_2	Ca^{2+}	$PLA_2 + Ca^{2+}$
Phosphatidylcholine	180.1 ± 32.7[a]	176.8 ± 28.5	171.8 ± 26.6	181.4 ± 30.0
Lysophosphatidylcholine	3.7 ± 1.6	3.3 ± 0.3	7.3 ± 2.4[c]	10.0 ± 1.8[c]
Phosphatidylethanolamine	127.2 ± 17.0	128.5 ± 16.3	127.6 ± 12.6	105.8 ± 13.8[b]
Lysophosphatidylethanolamine	4.4 ± 3.0	9.5 ± 4.9[b]	5.1 ± 1.3	34.2 ± 8.4[c]

The sarcolemmal fraction (1 mg protein/ml) was incubated at 37 C for 30 min at pH 7.0: with no addition; with PLA_2 (0.75 mg protein/ml); with calcium (5 nmol/ml); or with $PLA_2 + Ca^{2+}$.

[a]nmol/mg protein ± SD.

[b,c]Signify $P < 0.05$, 0.01, respectively, for significance of differences between incubated samples and incubated control. Quantities of other phospholipids (see ref. 13) were not altered significantly.

TABLE III

Major Fatty Acids and Alk-1-Enyl Ethers (mol %) of Choline and Ethanolamine-containing Lysophosphoglycerides of the Sarcolemma-enriched Fraction Formed by Incubation with PLA_2 and Ca^{2+}

Phospholipid	Incubation	Palmitic + Palmitoleic	Palmitoyl-1-enyl Ether	Stearic	Stearoyl-1-enyl Ether	Oleic	Linoleic	Arachidonic
PC[a]	Unincubated	14.8	20.4	8.7	0.7	22.2	17.0	15.3
LPC	37 C, PLA_2 + Ca^{2+}	32.9	19.2	21.9	0.5	15.3	8.2	2.7
PE	Unincubated	1.2	6.4	25.2	7.3	2.5	5.1	51.8
LPE	37 C, PLA_2 + Ca^{2+}	2.0	4.5	74.0	8.9	4.9	1.3	4.4

The membrane fraction (1 mg protein/ml) was incubated at 37 C for 30 min at pH 7.0 with PLA (0.75 mg protein/ml) in the presence of Ca^{2+} (5 nmol/ml).
Lysophospholipids were not sufficient in the unincubated preparation for fatty acid analysis.
[a]PC = phosphatidylcholine; LPC = lysophosphatidylcholine; PE = phosphatidylethanolamine; LPE = lysophosphatidylethanolamine.

DISCUSSION

We have shown previously (13) that the sarcolemma-enriched fraction has endogenous Ca^{2+}-stimulated phospholipase A activity and significant triacylglycerol lipase activity. In this study, we have characterized the specific changes in phospholipids and fatty acids, including arachidonic acid, following incubation with an exogenous Ca^{2+}-dependent PLA_2; there was no hydrolysis of triacylglycerols in the presence of the PLA_2. This observation may be related to the extreme cationic nature of the PLA_2 preparation (14). Also, no reduction was seen in the quantities of alk-1-enyl ethers in incubated phospholipid samples indicating no plasmalogenase activity. Thus, the total quantity of unesterified fatty acids (Table I, PLA_2 + Ca^{2+} minus control values) approached the total quantity of LPE plus LPC formed during 30 min of incubation (Table II, PLA_2 + Ca^{2+} minus control values). Also we found no loss of total phospholipid-P following incubation with PLA_2 plus Ca^{2+}; this provided evidence that lysophospholipase activity was insignificant during these incubations.

We examined the fatty acid and alk-1-enyl ether profiles of individual phospholipids of the membrane fraction both prior to incubation and following the combined activities of the endogenous and exogenous phospholipases (PLA_2 plus Ca^{2+}). Of particular note was the formation of 74% of the LPE as 1-stearoyl LPE whereas LPC was much more varied in composition including more esterified oleic and linoleic acids. However, both lysophospholipids contained less than 5 mol % of arachidonic acid. The sum of the mol % of arachidonic and linoleic acids of LPC or LPE are estimates of the minimum proportions of 2-acyl lysophospholipids; these may have been formed by the endogenous PLA_1 activity of this membrane preparation as indicated using radiolabeled PE (12). Only PC and PE were altered in composition during these incubations. It remains to be shown whether the preference of the exogenous PLA_2 for PE is dependent on some physical or biochemical parameters at the interface of PLA_2 with the membranes. Alternatively, the preference may be directed by the much higher arachidonic acid and lower palmitoyl-1-enyl ether content compared to PC (Table III).

These results suggest that the release of soluble PLA_2 from phagocytic cells would be expected to cause the formation of LPE and arachidonic acid as metabolites that may be important components of the process of injury to myocardial membranes in vivo.

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Cigarette Smoke Affects Lipolytic Activity in Isolated Rat Lungs

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ABSTRACT

Isolated perfused rat lungs liberated fatty acids at a rate of 15 μmol/hr during perfusion of triglyceride-rich medium through the pulmonary vascular bed. About 80% of this activity seemed to result from lipoprotein lipase and 20% to hormone-sensitive lipase. Ventilation of the lungs with cigarette smoke instead of air during the perfusion reduced fatty acid liberation by 23%. Pre-exposure of rats to cigarette smoke for either 1 or 10 days did not cause significant changes in lung lipolytic activity compared to sham-exposed controls.

INTRODUCTION

The lungs have an optimal location in the circulation to affect the levels of circulating substances because they have the largest capillary endothelial surface area in the organism and essentially all the blood is continuously passing through the pulmonary vascular bed. The lungs are rich in enzymes metabolizing both endogenous and exogenous substances and are considered a pharmacokinetically significant organ (1-5).

Pulmonary capillary bed is the first large endothelial surface with which chylomicrons come in contact after their appearance in the blood. Chylomicron triglycerides are attacked by extrahepatic lipoprotein lipases before the triglyceride-poor chylomicron remnants can be removed from the circulation by the liver (6). Lipoprotein lipase is an endothelial enzyme which is present in essentially all organs using fatty acids either for their energy demands or for the synthesis of lipid compounds (7). The first reports on pulmonary lipolytic activity were published in 1908 (8,9). Bragdon and Gordon have reported that some ^{14}C-triglyceride-labelled chylomicrons are taken up by rat lungs 10 min after intravenous injection of the substance (10).

Pulmonary lipoprotein lipase has gained little interest until recently. There are some reports on the variations of lipolytic activity of various tissues, including the lungs, caused by hypoxia, cold exposure, diets and exercise. Hamosh and Hamosh (11) have emphasized the nonresponsiveness of pulmonary lipoprotein lipase to fasting. This is in contrast to most other organs. It has been reported that cold exposure increases the pulmonary lipoprotein lipase activity in the rat (12). In this case, the increase was prevented by insulin administration. Of several hormones tested, only dexamethasone increased the pulmonary lipoprotein lipase activity (13). There also are reports stating that pulmonary lipolytic activity was increased by feeding rats sunflower seed oil (14) and by hypoxia (15). However, in experimental alloxan diabetes, the findings are somewhat controversial (13,15,16). Inhibition of pulmonary lipolysis by phenothiazine derivatives has been detected (17).

The incorporation of free palmitic acid in the lungs (18) and the production of surfactant (19) were unaffected by pretreatment of the rats with cigarette smoke. We have found previously that cigarette smoke exposure affects the pulmonary metabolism of foreign compounds (20,21), steroids (22), prostaglandins and angiotensin (23). In epidemiological studies (24), cigarette smoking is apparently connected with low HDL-cholesterol levels in the serum. The low serum HDL concentration has been shown epidemiologically to be related to pathogenesis of coronary heart disease (25). There are studies suggesting that changes in HDL-cholesterol levels might be caused by changes in the function of the lipoprotein lipase enzyme (26,27). The aim of this study was to investigate the effects of cigarette smoke on pulmonary lipolytic activity. Because of the lack of experience in using perfusion techniques for the study of lipid metabolism in lungs (28), we further developed our earlier methods so that they would be suitable for this purpose.

MATERIALS AND METHODS

Male adult Wistar rats (250-350 g) were used in these experiments. Cigarette smoke exposure was performed for either 1 or 10 consecutive days in an inhalation chamber, as described previously (29). The animals were exposed in these chambers to the smoke of 5 commercial cigarettes (0.9 mg nicotine and 18 mg tar per cigarette) daily for 1 hr. Sham-exposed animals were kept in similar chambers and exposed to air flow for corresponding times. Perfusion experiments with these pre-exposed animals were made 20 hr after the last exposure. One

group of animals was studied without prior exposure to cigarette smoke. During the perfusion, half of this group was ventilated with air only and another half with cigarette smoke for 2 min every 10 min and with air between cigarette smoke exposure. The apparatus for introducing smoke into the trachea was constructed from a Harvard piston pump generating to and fro movement. The generated flow was directed with 2 1-way valves, so that the lungs were ventilated with air (tidal vol 2 ml) pulled through a burning cigarette (Fig. 1).

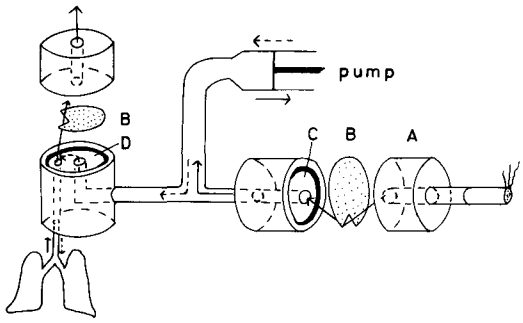

FIG. 1. A schematic representation of the machine used to introduce smoke into rat trachea. Harvard piston pump generates to and fro movement with tidal vol 2 ml. A: cigarette holder; B: rubber valves; C: hollow (1 mm deep) allowing opening of the valve; D: hollow (1 mm deep) containing the rubber valve. Solid arrows show the flow of smoke during the suction period of the pump and dotted arrows represent the flow during the pushing of the pump. The blocks containing the valves were made from nylon and they were connected with silicone tubing.

Lung Perfusion System

A summary of our method for perfusing isolated rat lungs (30) follows. After the rats were anesthetized with pentobarbital (50 mg/kg, Nembutal®), the trachea was connected to a pressure-regulated respirator. Because preliminary experiments without heparin caused poor perfusion of the lungs, we injected 500 IU of heparin into the pulmonary circulation after thoracotomy. This treatment is known to cause a minor liberation of pulmonary lipoprotein lipase enzyme (11). The pulmonary artery was cannulated via the right ventricle with a polyethylene tube and the ventricles were excised. The lung block was removed and connected to the perfusion apparatus. The preparation was perfused with Krebs bicarbonate solution containing 1 g/ℓ glucose and 4.5 g/ℓ albumin. The perfusions were made at 37 C with a pulsatile flow of 10 ml/min and a pressure of 17/21 mm Hg.

Commercial triglyceride emulsion (Intralipid®, Vitrum AB, Sweden, 200 mg/ml) was used as a substrate. Preceding the perfusion, 0.35 ml or 3.5 ml of Intralipid® was mixed with Krebs solution (final vol 70 ml) to give a final concentration of 1.1 mmol/ℓ or 11.3 mmol/ℓ of triglyceride. The higher triglyceride level was then chosen in this series to ensure substrate saturation (see Results, Table I). During recirculating perfusions, 1-ml samples were withdrawn from the reservoir and the amount of free fatty acid was analyzed using the Mikac-Devic spectrophotometric method (31). The amount of free fatty acid in the medium before perfusion was subtracted and the results were corrected for the sample volumes. To evaluate the possible significance of hormone-sensitive lipase as speculated by Hamosh and Hamosh (11), additional experiments were made with lungs perfused using Krebs solution but without triglyceride. To estimate the rate of free fatty acid liberation resulting from heparin-releasable enzyme (11),

TABLE I

The Effect of Triglyceride Concentration on the Liberation of Fatty Acids by Isolated Perfused Rat Lungs[a]

Perfusion time (min)	11.3 mmol/ℓ	1.1 mmol/ℓ	0 mmol/ℓ
20	8.9 ± 1.0	7.3 ± 1.8	3.2 ± 0.4
40	15.5 ± 2.9	14.2 ± 3.5	5.6 ± 0.2
60	22.5 ± 2.1	18.6 ± 3.8	6.6 ± 0.9
80	26.8 ± 3.7	24.0 ± 3.8	7.1 ± 1.1
100	30.9 ± 2.8	27.9 ± 4.8	7.3 ± 0.7
120	34.6 ± 3.2	28.3 ± 7.1	8.3 ± 1.0
Number of experiments in each series	4	5	4

[a]The values are expressed as μmol fatty acid liberated at perfusion times indicated, mean ± SEM.

we did control experiments, removing the lungs after 5 min perfusion (11.3 mmol triglyceride concentration). The perfusion medium was thereafter circulated for 115 min without the lungs.

RESULTS AND DISCUSSION

When the medium contained 11.3 mmol/ℓ triglycerides, perfused rat lungs hydrolyzed from it free fatty acids (FFA) at a rate of 15 μmol/hr (Table I). The use of a physiological concentration of triglyceride, 1.1 mmol/ℓ, resulted in only a slight decrease in the rate of fatty acid liberation (13 μmol/hr). Since substrate saturation was necessary, the higher concentration was chosen in further experiments. The results were surprisingly similar to the reported levels of the lipoprotein lipase activity in lung extracts ranging from 10 to 15 μmol FFA released/g/hr (11,32), since the lungs of our rats weighed ca. 1 g. Fatty acid liberation was linear during the 2-hr perfusion period (Fig. 2). These findings point to a higher lipolytic activity/tissue wt in the rat than in the rabbit, as Heineman (28), has reported about similar amounts of triglyceride to be hydrolyzed by rabbit lungs in perfusion, whereas rabbit lungs weigh considerably more.

The triglyceride concentration used in this study (11.3 mmol/ℓ) exceeded normal plasma triglyceride levels. Our control results (Table I) using a physiological concentration of triglyceride (1.1 mmol/ℓ), resulted in 37% hydrolysis of added triglyceride in 2 hr. Since the perfusion flow in this study was significantly lower than the cardiac output in rats (30), the lungs could theoretically account for a large proportion of triglyceride catabolism in the organism.

Hamosh has criticized earlier perfusion experiments (28) for not taking into consideration the hormone-sensitive lipase activity (11). Our experiments without added triglyceride resulted in linear fatty acid liberation from the lungs to the perfusion medium (Fig. 2). This activity, attributed to the hormone-sensitive lipase using tissue triglycerides, corresponded to 17% of the overall lipolytic activity. No corrections for this activity were made in the results. It has been reported (11) that 10% of the pulmonary lipoprotein lipase activity is liberated from perfused lungs within 30 min after heparin administration. When rat lungs were removed after 5 min perfusion from the circulation, fatty acids were released in the perfusion medium during the following 115 min at a rate of 5 μmol/hr in this study. This amount is as much as 33% of the rate of triglyceride hydrolysis in 120-min lung perfusions.

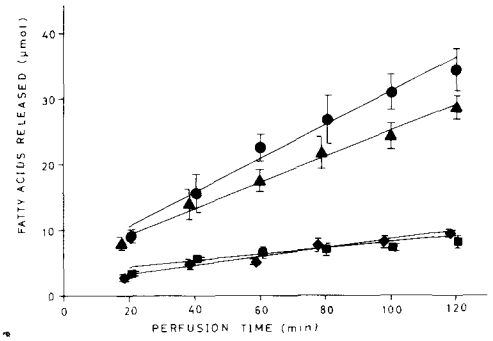

FIG. 2. The effect of acute smoke ventilation during perfusion on pulmonary lipolytic activity. Isolated rat lungs were perfused with either triglyceride-containing (11.3 mmol/ℓ) medium or with a medium without added triglycerides. ● = Control perfusion with Intralipid®, ventilation with air (n=4); ▲ = acute smoke ventilation during perfusion with Intralipid® (n=5); ■ = control perfusion without triglyceride (n=4); ♦ = acute smoke ventilation during perfusion without triglyceride (n=3).

The released enzyme is continuously in contact with substrate in our system. This explains why the enzyme washed out in 5 min causes such a high metabolism compared to the 10% enzyme activity reported to be released from the lungs in 30 min (11).

When the lungs were ventilated with cigarette smoke during perfusion (Fig. 2), a statistically significant decrease (23%) in lipolytic activity was seen when compared to air-ventilated lungs ($2P < 0.05$, Student's t-test). This decrease probably resulted from a general effect of cigarette smoke on metabolism as we have detected a similar inhibition in the metabolism of foreign compounds during smoke ventilation (unpublished results). The lipolytic activity attributed to hormone-sensitive lipase (capable of using tissue triglycerides) was not affected by smoke ventilation (Fig. 2).

Pre-exposure of the rats to cigarette smoke 20 hr before the perfusion did not affect the pulmonary lipolytic activity (Fig. 3). Despite the tendency to produce larger amounts of free fatty acids in the medium, neither of the animal groups exposed to smoke for 10 days differed statistically from its sham group (Fig. 4). On the other hand, when 1-day and 10-day smoke-exposed groups were compared, there was a significant difference between the groups ($2P < 0.05$, Student's t-test). One possible explanation for the increased lipolytic activity in animals treated in the exposure system for longer periods might be that the treatment of the animals affects pulmonary metabolic activity, nonspecifically via hormonal or

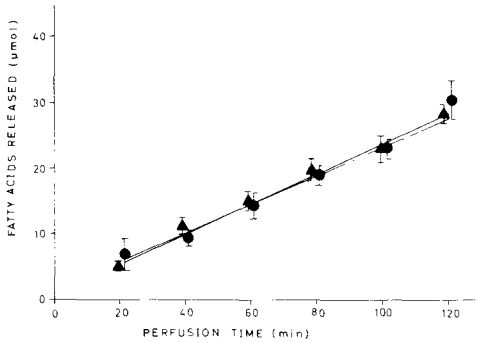

FIG. 3. The effect of 1 hr pre-exposure of the rats to the smoke of 5 filter cigarettes 20 hr before perfusion on pulmonary lipolytic activity. The lungs were perfused with triglyceride-containing medium (11.3 mmol/ℓ). ● = Smoke-exposed rats (n=9); ▲ = sham-exposed rats (n=6).

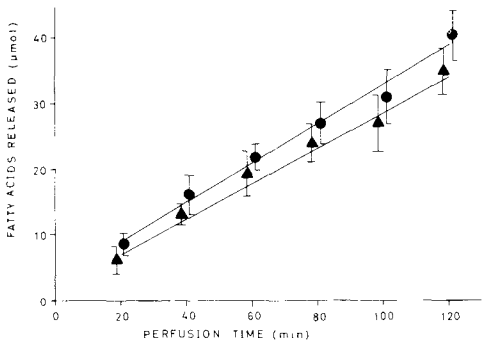

FIG. 4. The effect of 1 hr pre-exposure of the rats to the smoke of 5 filter cigarettes during 1 hr for 10 consecutive days on pulmonary lipolytic activity. The lungs were perfused with triglyceride-containing medium (11.3 mmol/ℓ). ● = Smoke-exposed rats (n=6); ▲ = sham-exposed rats (n=4).

nervous mechanisms. Such mechanisms may be elicited by the stress caused by altered conditions alone, as there was also a slight difference in the lipolytic activities between 1- and 10-day sham groups.

Although it may not be justified to relate these results to the effects of cigarette smoking on lipid kinetics in man, there are reports (24,33) of altered lipid levels in smokers. Because smoke ventilation seems to inhibit lung metabolism, it is tempting to attribute this phenomenon to toxic metabolic effects of inhaled smoke (34). Many of the compounds present in inhaled cigarette smoke are readily transferred to circulating blood. Consequently, inhibition of lipoprotein lipase also is possible in vascular beds outside the lungs.

ACKNOWLEDGMENTS

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Singlet Oxygen Oxidation of Methyl Linoleate: Isolation and Characterization of the $NaBH_4$-reduced Products[1]

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ABSTRACT

The mixture of diene hydroperoxides from methylene blue-sensitized oxidation of methyl linoleate was reduced with $NaBH_4$ and the resulting alcohols were separated by high pressure liquid chromatography (HPLC). Four diene alcohols were isolated in approximately equal yields from adsorption and reversed phase HPLC; the isomers were identified as methyl esters of 9-hydroxy-10,12-, 10-hydroxy-8,12-, 12-hydroxy-9,13- and 13-hydroxy-9,11-octadecadienoate. Formation of equal yields of both conjugated and nonconjugated diene alcohols from methyl linoleate is characteristic of singlet oxygen oxidations. The detection of the easily separated nonconjugated isomer methyl 10-hydroxy-*trans*-8,*cis*-12-octadecadienoate from methyl linoleate is proposed as a test to probe the involvement of singlet oxygen in biological oxidations.

INTRODUCTION

We have a long standing interest in the oxidations of polyunsaturated fatty acids (PUFA) as models for in vivo processes that cause cell death (1-4). Recently we began an investigation of the mechanism of the xanthine oxidase-induced peroxidation of PUFA. This oxidation has been shown to require superoxide ion as an intermediate, although superoxide ion does not react directly with PUFA. Previous researchers have shown that superoxide ion undergoes reactions that produce other species which initiate the autoxidation of PUFA; these reactions include those that yield radicals and/or singlet oxygen (4-10). We therefore wished to determine whether singlet oxygen plays a role in xanthine oxidase-induced PUFA peroxidation. As an alternative to the Teng and Smith method (11) (which uses cholesterol), we have developed a test that distinguishes between the singlet oxygen and autoxidation products from PUFA.

Singlet oxygen has been shown to react with linoleate to yield a mixture of conjugated and nonconjugated diene hydroperoxides (12-17). In contrast, nonconjugated diene hydroperoxides have not been detected from the autoxidation of linoleate (18-20); this free radical chain process would be expected to yield only the conjugated 9- and 13-hydroperoxides. Therefore, singlet oxygen oxidations can be detected by the production of nonconjugated dienes that are not formed by autoxidation. In this paper we describe the isolation and characterization of the alcohols produced by $NaBH_4$-reduction of hydroperoxides formed by dye-sensitized photooxidation of methyl linoleate and the conditions under which both conjugated and nonconjugated diene alcohols can be reproducibly separated and analyzed by high pressure liquid chromatography (HPLC).

MATERIALS AND METHODS

Methyl linoleate (99%) was obtained from Calbiochem (La Jolla, CA) and purified by liquid column chromatography on silica gel using 10% ether in hexane as eluant. Methylene blue chloride from Aldrich (Milwaukee, WI) was recrystallized from methanol/water before use. Soybean lipoxidase (E.C. 1.13.1.13) was obtained from Sigma (St. Louis, MO). All solvents were reagent grade. Hexane was stirred with sulfuric acid, washed, dried and then distilled through a glass helices packed column.

Spectra were recorded on a Cary 118 ultraviolet (UV) -visible spectrometer, on Perkin-Elmer 621 and Beckman IR-9 infrared (IR) spectrometers (in $CDCl_3$), on a Varian HA-100 nuclear magnetic resonance (NMR) spectrometer (in $CDCl_3$), and on Hitachi Perkin-Elmer MS-4 and Dupont 21-491 mass spectrometers (operated at 70 v).

Chromatography

Liquid chromatography was performed on a Waters ALC 202 liquid chromatograph (Waters Associates, Milford, MA), equipped with a Waters M-6000 chromatography pump. Adsorption chromatography was carried out on a 30 cm x 3.9 mm Waters μ-Porasil column using 0.3% 2-propanol in hexane as the solvent.

[1] A preliminary report of these results was presented at the 177th meeting of the American Chemical Society, Honolulu, HI, April 1-6, 1979; see abstracts of papers, paper No. ORGN-375.

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Reversed phase chromatography was conducted on a 30 cm x 1 cm Waters C_{18} μ-Bondapak column using 54% ethanol/46% water (v/v) as the solvent. Analytical thin layer chromatography (TLC) was carried out on 5 cm x 20 cm Brinkmann Silica Gel G coated plastic sheets containing fluorescor. The analysis of ozonolysis products was performed on a Varian Associates Model 2400 FID gas chromatograph equipped with a 1/8-inch glass column containing 10% DEGS on Chromosorb W.

Photooxidation

Methyl linoleate (38 mM) and methylene blue chloride (0.15 mM) dissolved in methanol (100 ml) were placed in an apparatus similar to that described by Higgins et al. (21). The solution was sparged with oxygen, cooled to 10 C, then irradiated for 3.6 hr by a 650 watt DWY tungsten-halogen lamp operated at 50 volts. After photolysis the solution was cooled in an ice-water bath and treated with excess $NaBH_4$ (100 mg). The solvent was evaporated, the residue treated with 40 ml of 0.5 N aq HCl, extracted with 50% ether/hexane (X2, 100 ml), dried over Na_2SO_4 and then evaporated. When it was desirable to isolate the linoleate hydroperoxides, the methanol was evaporated, the residues taken up in hexane, filtered through a 10 cm x 2.5 cm column of Celite to remove methylene blue and then fractionated on a column of Silica Gel G. Unreacted methyl linoleate was eluted with 5% ether/hexane and the linoleated hydroperoxides eluted as a single band with 50% ether/hexane.

Preparation of Methyl 13-Hydroperoxy-*cis*-9,*trans*-11-octadecadienoate

A solution of linoleic acid (8 mM, 100 ml) in 0.05 M boarate buffer (pH = 9) and 0.15 M in ethanol was cooled to 0 C and sparged with oxygen. To this solution was added a soybean lipoxidase solution giving a final enzyme concentration of 4050 units/ml. (The assay procedure used was that described by Sigma.) After 10 min, the solution was acidified to pH 4, then extracted with 20% ether/hexane (X3, 90 ml). The solvent was evaporated at 24 C, the residues dissolved in 10 ml methanol, cooled in an ice-water bath, then treated with 20 mg of $NaBH_4$. After 1 hr, 30 ml of 0.2 N aq HCl was added and the solution extracted with 20% ether/hexane (X2, 40 ml). The solvent was evaporated, the residues taken up in ether and esterified at 0 C with diazomethane.

RESULTS

Methylene blue-sensitized photooxidation of methyl linoleate gives a polar residue which moves as one spot on TLC (R_f = 0.45) using 50% petroleum ether/ether. This material gave a positive peroxide test with N,N-dimethylphenylenediamine sulfate. Reduction with methanolic $NaBH_4$ gave a nonperoxidic material which moved as one spot on TLC (R_f = 0.33). The residue was analyzed by adsorption HPLC using a refractive index (RI) detector and found to contain 3 major fractions (A,B and C), 2 of which were also detected by a 254 nm detector (A and C), and 2 minor ones (D and E). Fractions A, B and C were isolated. Recycle chromatography of fraction A on a reversed phase HPLC column yielded components A1 and A2 after 3 cycles (Fig. 1).

The various isomers were characterized using the combined results of IR, NMR, UV, mass spectra (MS) and ozonolysis. The IR spectra of A2 and C show olefinic absorption bands between 3013-3010 cm^{-1} and 2 absorption bands (987-980 cm^{-1} and 951-946 cm^{-1}) suggesting that both isomers contain conjugated *cis,trans* double bonds (10,13-15,18,22). Bands for hydroxy (3625-3617 cm^{-1}) and ester moieties (1743-1741 cm^{-1}) are consistent with a hydroxy-containing linoleate. The UV spectra of A2 and C with λ_{max} at 235 nm support

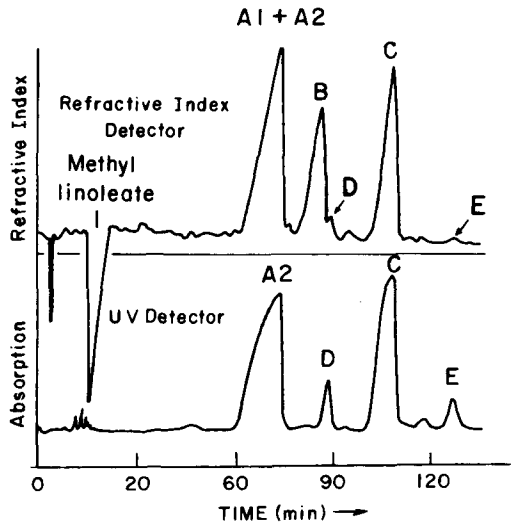

FIG. 1. HPLC trace of methyl linoleate alcohol isomers separated on a 30 cm x 3.19 mm μ-Porasil column using 0.3% 2-propanol in hexane as eluant. Upper trace, refractive index (RI) detector; lower trace, ultraviolet (UV) detector. A1: methyl 12-hydroxy-*cis*-9,*trans*-13-octadecadienoate; A2: methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate; B: methyl 10-hydroxy-*trans*-8,*cis*-12-octadecadienoate; C: methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate; D and E: small quantities of the *trans,trans* isomers of A2 and C.

the IR assignment of a *cis,trans* conjugated diene, but not a *trans,trans* conjugated diene (λ_{max} = 232-233 nm) (18,23,24). The NMR spectra of A2 and C also support the IR analysis with signals for olefinic protons at 6.48-6.54 ppm (m) and 5.67-5.70 ppm (m), the methine proton of the carbinol moiety at 4.16-4.15 ppm (q), and allylic absorptions at 2.21 ppm (t). The integration ratios of the proton signals are consistent with these assignments. These NMR spectra are in agreement with those reported in the literature (25,26). Isomer A2 has the same HPLC retention volume as methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate and C has the same retention volume as methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate (isolated along with the 13-hydroxy-*cis*-9,*trans*-11 isomer and both *trans,trans* isomers from $NaBH_4$-reduced, autoxidized methyl linoleate). The minor components D and E, which constitute ca. 2% of the products, have retention volumes that suggest they are the *trans,trans* conjugated diene alcohols methyl 13-hydroxy-*trans*-9,*trans*-11-octadecadienoate and methyl 9-hydroxy-*trans*-10,*trans*-12-octadecadienoate, respectively.

The IR spectra of A1 and B have bands at 3010-3013 cm^{-1} and a single absorption band at 971-972 cm^{-1} suggesting that both isomers contain an isolated double bond (13,14,18,22). Bands for hydroxyl (3617-3620 cm^{-1}) and an ester moiety (1747-1739 cm^{-1}) are consistent with a reduced, oxidized linoleate. The NMR of A1 and B support the IR analysis showing olefinic absorptions at 5.55-5.59 ppm (m), the methine proton of the carbinol moiety at 4.08-4.11 ppm (q), and allylic absorptions at 2.05 ppm (m) and 2.20-2.28 ppm (a broad triplet overlapping with the absorption of protons located adjacent to the ester carbonyl). Integration of the NMR yields values consistent with these assignments. The NMR spectra of conjugated diene alcohol A2 and nonconjugated diene alcohol A1 are compared in Figure 2. Differences between these isomers are most pronounced in the olefinic region. Isomers B and C have NMR spectra similar to A1 and A2, respectively. Mass spectral analysis of A1 and B revealed molecular ions at 310 which corresponds to a C_{18} methyl ester having double bonds and 1 hydroxy moiety. In addition, the MS of A1 and B show strong ions at m/e 113 and 199, respectively. These ions correspond to fragments having formulas $C_7H_{13}O^+$, resulting from cleavage adjacent to the hydroxy group, as depicted in Figure 3. This proves that A1 has

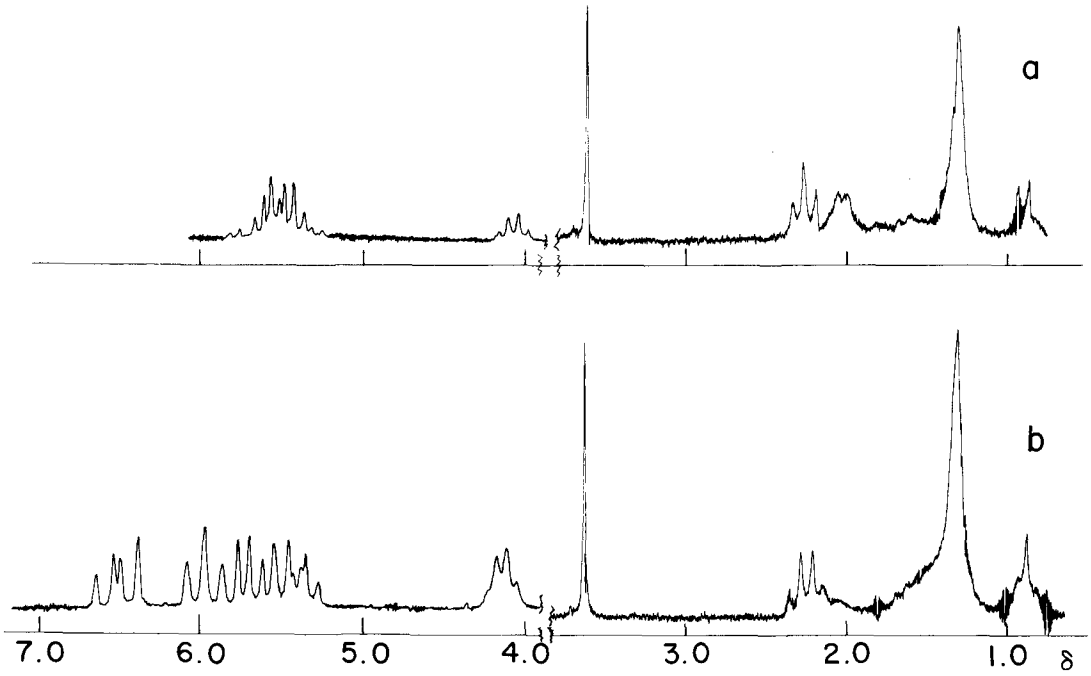

FIG. 2. Nuclear magnetic resonance spectra of 2 diene alcohols. (a) Compound A1: methyl 12-hydroxy-*cis*-9,*trans*-13-octadecadienoate; (b) Compound A2: methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate. Spectra of the 7 to 4 δ region were accumulated from 50 (8 min) scans of the diene alcohols (3-4 mg) in $CDCl_3$-TMS solution using a Varian CAT.

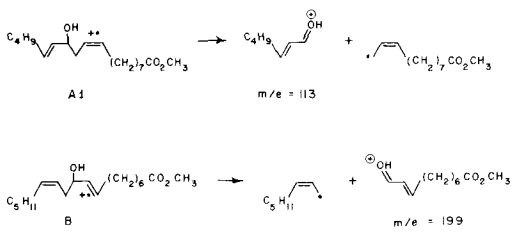

FIG. 3. Mass spectrum fragmentation which yields the m/e = 113 and m/e = 199 ions.

the hydroxy at C-12 and B has it at C-10. Each fragment retained only 1 of the 2 double bonds, implying that the hydroxy lies between the double bonds. Ozonolysis of A1 and B in the presence of BF_3/methanol (27) gives the diesters dimethyl azelate and dimethyl suberate, indicating that the double bond closest to the ester moiety is between C-9 and C-10 in A1 and C-8 and C-9 in B.

The relative yields of B and C were determined by adding an aliquot of methyl ricinoleate as internal standard to the $NaBH_4$-reduced photooxidation mixture, then separating the components on a μ-Porasil column using an RI detector to monitor the separation. The response factors for B (0.75) and C (1.06) relative to methyl ricinoleate were determined by co-injection of weighed quantities of B and C with methyl ricinoleate. The B/C ratio relative to methyl ricinoleate was found to be 0.92 ± 0.08. Assuming the B/C ratio is identical to the A1/A2 ratio, the nonconjugated alcohols constitute 46 ± 4% of the products.

DISCUSSION

Photooxidation of methyl linoleate followed by $NaBH_4$ reduction yields approximately equal amounts of 4 isomeric diene alcohols; 2 have conjugated double bonds and 2 have isolated double bonds. Formation of approximately equal amounts of conjugated and nonconjugated diene alcohols is consistent with previous reports in the literature on this sytem (14-17). Structures of the 4 diene alcohols are given in Figure 4. Ozonolysis confirmed the position of the double bond closest to the ester moiety in both A1 and B. The positions of the remaining double bonds were deduced in the following manner. Isomer B, which is formed by singlet oxygen oxidation of the C-9,10 double bond of linoleate, retains the *cis* C-12,13 double bond intact. Oxidation of the C-12,13 linoleate double bond introduces oxygen at C-12, yielding A1. Since the singlet oxygen ene reaction proceeds as if it were a thermally allowed concerted reaction, the position of the

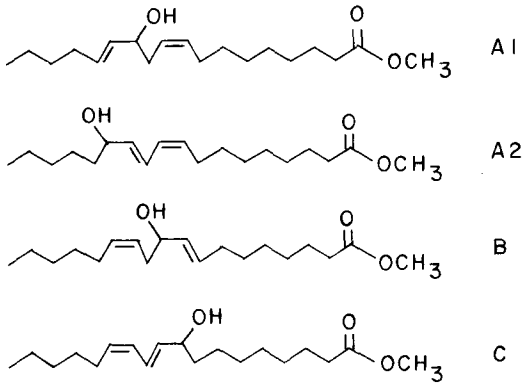

FIG. 4. Diene alcohols produced by $NaBH_4$ reduction of the hydroperoxides formed from methylene blue-sensitized photooxidation of methyl linoleate: A1: methyl 12-hydroxy-*cis*-9,*trans*-13-octadecadienoate; A2: methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate; B: methyl 10-hydroxy-*trans*-8,*cis*-12-octadecadienoate; C: methyl 9-hydroxy-*trans*-10, *cis*-12-octadecadienoate.

hydroxy establishes the position of the remaining double bond in nonconjugated A1 to lie between C-13 and C-14.

Foote and coworkers (21,28) have noted that the product distribution from the singlet oxygen ene reaction is not particularly sensitive to the number of hydrogens available for reaction or to a substituent directing effect. We and others (14-17,29) have observed that 1,4-dienes (like linoleate) react with singlet oxygen yielding approximately equal amounts of conjugated and nonconjugated products and Chan (30) reports that linolenate reacts with singlet oxygen to give a nearly statistical distribution of conjugated (69%) and nonconjugated (31%) hydroperoxides. From these observations, we conclude that doubly allylic protons are not appreciably more susceptible to attack than are singly allylic protons. Moreover, the shifted double bond appears to be predominantly, if not exclusively, *trans*. This high degree of stereoselectivity in the reaction of singlet oxygen with linoleate has not been previously demonstrated, but it is consistent with reports describing the subtle configurational features that influence the course of olefin-singlet oxygen reactions (31-34). (However, 2 recent reports show that extensive irradiation of these peroxide solutions will result in changes in the isomer ratios [16,29]; undoubtedly this is the result of excited-state dye-hydroperoxide interactions.) Figure 5 rationalizes the reactions of singlet oxygen with linoleate in terms of steric interactions; the conformation of lowest energy (least steric crowding) is preferentially oxidized.

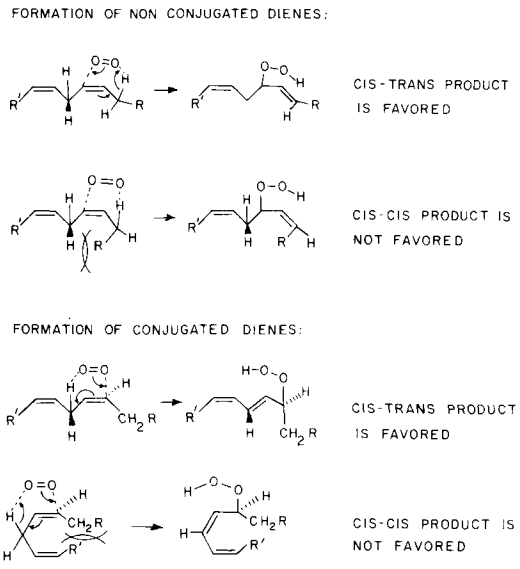

FIG. 5. Conformations for the ene reaction of singlet oxygen with the 1,4-diene moiety of methyl linoleate. The *cis,trans* isomer is predicted to be favored over the *cis,cis* for both the conjugated and nonconjugated isomers.

Of the 4 products formed in the singlet oxygen oxidation of linoleate, the nonconjugated diene alcohol B, methyl 10-hydroxy-*trans*-8,*cis*-12-octadecadienoate, is most easily separated by HPLC from the other *cis,trans* and *trans,trans* conjugated diene alcohols in the $NaBH_4$-reduced product mixtures. Thus, HPLC identification of this isomer demonstrates singlet oxygen participation in a linoleate oxidation. This procedure will allow linoleate to be used as a probe for singlet oxygen participation in biological lipid oxidations.

ACKNOWLEDGMENTS

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Composition of Plasma and Nascent Very Low Density Lipoprotein from Perfused Livers of Hypercholesterolemic Squirrel Monkeys

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ABSTRACT

The composition of circulating very low density lipoprotein (VLDL) was compared with the composition and secretion of nascent VLDL from perfused livers of squirrel monkeys that were fed unsaturated or saturated fat diets to elicit different degrees of plasma hypercholesterolemia. All squirrel monkeys studied had cholesteryl ester-rich plasma VLDL, although greater enrichment occurred in hypercholesterolemic animals fed saturated fat. Livers from hypercholesterolemic animals were capable of secreting VLDL particles enriched in cholesteryl ester, suggesting hepatic origin for a portion of this circulating lipid moiety. Total VLDL lipid, but not protein output by perfused livers of hypercholesterolemic monkeys, was greater than that by livers from hypocholesterolemic animals. These results indicate that saturated fat-induced hypercholesterolemia is associated with changes in the composition of hepatic VLDL in the squirrel monkey.

INTRODUCTION

Plasma lipid and lipoprotein profiles of most mammals, including nonhuman primates, generally reveal negligible quantities of plasma triacylglycerol and associated very low density lipoprotein (VLDL). However, dietary consumption of saturated fat and/or cholesterol have resulted in the circulation of cholesteryl ester-rich VLDL in rats (1), guinea pigs (2,3), rabbits (4,5), dogs (6) and in both Old and New World monkeys (7-9). Previous study of cebus and squirrel monkeys (10) indicated that the primary expansion of the cholesteryl ester pool during saturated fat feeding was associated with high density lipoprotein (HDL) in cebus and with low density lipoprotein (LDL) in squirrel monkeys. In fact, VLDL played only a minor role in the total cholesterolemia. Nevertheless, since cholesteryl ester-rich VLDL may be unusually atherogenic (11) their origin and metabolism are of interest.

A fundamental question related to the metabolism of such particles is whether they are cholesteryl ester-rich when secreted or become enriched during prolonged circulation through transfer of cholesteryl ester from other lipoproteins and selective removal of triacylglycerol. If secreted, do they originate primarily from liver or intestine? For example, in cholesterol-fed rabbits, intestinally derived chylomicron remnants appear to be a major source of plasma VLDL cholesteryl esters (4). On the other hand, perfused livers of hypercholesterolemic guinea pigs fed cholesterol (3) and rats made hypercholesterolemic with cholesterol and propylthiouracil feeding (12) secrete cholesteryl ester-rich VLDL, suggesting hepatic origin for these particles. In vivo studies in squirrel monkeys made hypercholesterolemic with saturated fat and cholesterol, and which circulated cholesteryl ester-rich VLDL, concur with the findings in guinea pigs and rats, but also suggest that a portion of the cholesteryl ester moiety could be derived from nonhepatic sources such as plasma lecithin: cholesterol acyl transferase (LCAT) (13) and the intestine (14).

Similar to other lipoproteins, the concentration of VLDL particles is determined by their rates of production and catabolism. However, there is disagreement as to whether hypercholesterolemia is associated with increased or decreased hepatic VLDL secretion. Several in vivo studies with Triton blockade have indicated that triglyceride (15,16) and VLDL (14,17) secretion rates are depressed during hypercholesterolemia. However, isolated perfused livers from hypercholesterolemic rats (18,19), guinea pigs (3) and gerbils (20) secrete more VLDL than hypocholesterolemic animals.

These observations raised the following questions which were investigated in squirrel monkeys: (a) do cholesteryl ester-enriched VLDL particles circulate in the plasma of nonhuman primates fed saturated fat without added dietary cholesterol; (b) can the liver secrete a nascent VLDL particle reflecting the cholesteryl ester-enriched composition of those in circulation; and (c) does the quantity of VLDL secreted by the isolated, perfused liver in the squirrel monkey correspond with the amount of VLDL and triacylglycerol secreted in vivo after Triton blockade, i.e., is it de-

pressed in livers from monkeys with high levels of circulating cholesterol?

METHODS

To evaluate the enrichment of VLDL with cholesteryl ester resulting from saturated fat feeding, 4 hypercholesterolemic monkeys (*Saimiri sciureus,* Letician), born and raised in our primate nursery, were selected from a larger group of monkeys fed 10% coconut oil or lard (saturated fat) and compared with 4 hypocholesterolemic monkeys selected from those fed 10% corn oil or cottonseed oil (unsaturated fat) as the fat component of a semipurified diet for a period of 3 to 4 years from birth. The diet composition was as follows (g/100 g): casein, 20.0; dextrose, 40.7; coconut oil, corn oil, lard or cottonseed oil, 10.0; salt mix, 4.0 (21); vitamin mix, 2.0 (21); choline chloride, 0.3; cellulose, 23.0. Body weight ranged from 620-920 g with a mean ± SE of 758 ± 27.

After an 18-hr fast, animals were anesthetized with sodium pentobarbital (30 mg/kg body weight) and 10 ml of blood collected from the inferior vena cava for plasma VLDL isolation. Plasma VLDL ($d < 1.006$) was isolated by ultracentrifugation (22) for 20 hr at 40,000 rpm in a Beckman 40.3 fixed angle rotor and Model L ultracentrifuge. After centrifugation, the top 1 ml of each tube (VLDL fraction) was collected by aspiration and dialyzed against 0.15 M NaCl for 48 hr. Aliquots of the dialysate were analyzed for protein (23), total cholesterol (24) and phospholipid (25). The remaining dialysate was extracted for neutral lipids (26), separated into the various lipid classes by thin layer chromatography (TLC) and quantitated by in situ TLC charring densitometry (27). Electrophoresis of VLDL was carried out on agarose gels (28) and stained with Oil-Red-O.

To assess hepatic secretion of VLDL, the bile duct and portal vein were cannulated prior to initial perfusion of the liver with Krebs-Ringer bicarbonate buffer (KRB) containing 2% albumin. The liver was then removed from the animal, placed in a modified Miller apparatus (29) and perfused with 125 ml of a recirculating cell-free medium containing: (per 100 ml), an amino acid mixture (Eagle's Minimum Essential Amino Acids and glutamine, Microbiological Associates, Inc., Bethesda, MD); 2% fatty acid-poor albumin (Cohn Fraction V, Armour Pharmaceutical, Montgomery, IL); 360 mg glucose; and 5 mg streptomycin and penicillin. Perfusion was by gravity flow at a flow rate of 60 ml/min with a hydrostatic pressure of 15 cm of water. All perfusions lasted 3 hr, during which time the media was constantly equilibrated with a humidified gas phase of 95% O_2 and 5% CO_2. Liver viability was judged by gross appearance, perfusion flow rate, bile flow, serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) (Sigma Reagent Kits, St. Louis, MO). Postperfusion livers ranged from 13.8 to 21.7 g with a mean ± SE of 17.8 ± 0.8 g.

After 3 hr, the perfusate (ca. 90 ml) was concentrated at 4°C to ca. 25-30 ml by ultrafiltration at 20-25 psi in an Amicon Concentrating Cell (Model 52) using PM-30 membranes. The VLDL fraction was isolated by ultracentrifugation as already described (22). Procedures for agarose electrophoresis and measurements of protein, total lipid and the various lipid classes of perfusate VLDL were the same as those already described for plasma lipids and VLDL.

RESULTS

Plasma Cholesterol and Lipoprotein Profile

Monkeys designated as hypercholesterolemic and fed saturated fat as coconut oil (n = 2) or lard (n = 2), had a mean plasma cholesterol concentration of 273 ± 15 mg/dl. Four monkeys designated as hypocholesterolemic and fed unsaturated fat as corn oil (n = 3) or cottonseed oil (n = 1) had a mean plasma cholesterol value of 113 ± 6 mg/dl. Agarose gel electrophoresis of plasma indicated that hypercholesterolemic monkeys experienced a substantial increase associated with the LDL-VLDL band which was verified by electrophoresis of isolated VLDL (Fig. 1).

Plasma VLDL

The composition of plasma VLDL differed in monkeys depending on whether their plasma cholesterol values were high or low (Table I). The relative protein content, which was similar between the 2 groups of monkeys, was slightly greater than previously published data (30) because of contamination with albumin, since the VLDL were not washed by a second centrifugation. Since lipid analyses were our major concern, the washing step, which reportedly results in loss of lipoprotein (31), was omitted to ensure the greatest possible yield of the small quantity of plasma VLDL usually found in monkeys. A greater percentage of cholesteryl ester (40 vs 27) and slightly less triacylglycerol (31.5 vs 23.5) and phospholipid were observed in plasma VLDL of hypercholesterolemic monkeys when compared to hypocholester-

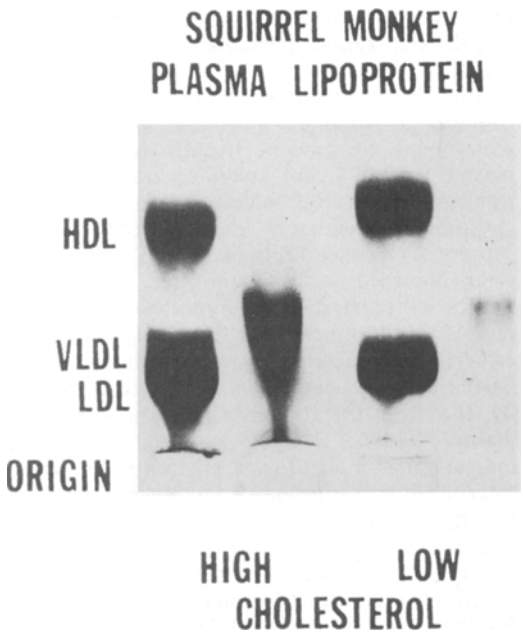

FIG. 1. Agarose electrophoresis depicts plasma lipoproteins from squirrel monkeys with high and low plasma cholesterol concentration. The VLDL fraction from each was separated by ultracentrifugation to demonstrate the substantial increase in that density in the hypercholesterolemic monkey.

olemic animals, although VLDL from the hypocholesterolemic group also contained a relatively high concentration of cholesteryl esters.

Perfusate VLDL

The viability of livers was assessed by several physiological parameters such as bile flow and transaminase release by the liver (SGOT and SGPT). At the end of 3 hr, values were similar to those reported by Bartosek et al. (32) for rats: bile output (ml/hr), 0.51 ± 0.10; SGOT and SGPT (mμ/ml/hr), 9.3 ± 1.8 and 2.0 ± 0.4, respectively. Ultrastructural assessment of the liver (not shown) indicated that perfusion did not disrupt normal cell organization, and lipoproteins were visualized in the Golgi apparatus and associated vesicles adjacent to the space of Dissé. Similar to results in the rat (12,33), the VLDL released into the perfusate migrated more slowly on agarose electrophoresis than plasma VLDL (Fig. 2).

Because perfusate VLDL composition was to be compared to plasma VLDL, the washing step was again omitted so that direct comparisons could be made. Independent of dietary fat and serum cholesterol concentration, perfusate VLDL contained relatively more triacylglycerol than plasma VLDL and relatively less cholesterol which was 75-90% esterified (Table I). Perfusate VLDL from hypercholesterolemic monkeys contained relatively less protein and more lipid than either the perfusate VLDL from hypocholesterolemic monkeys or plasma VLDL. Livers from hypercholesterolemic monkeys apparently secreted less dense VLDL particles than those from the low cholesterol group as evidenced by the secretion of significantly more VLDL total lipid, esterified cholesterol, triacylglycerol and phospholipid in the hypocholesterolemic group, whereas net VLDL protein secreted was essentially identical (Table II).

DISCUSSION

From these data and those of Illingworth (30), plasma VLDL from squirrel monkeys under normal dietary circumstances apparently is cholesteryl ester-rich (27% total lipoprotein)

TABLE I

Plasma and Perfusate VLDL Composition from Hypo- and Hypercholesterolemic Squirrel Monkeys

VLDL component	Plasma VLDL		Perfusate VLDL	
	Hypo	Hyper	Hypo	Hyper
	Percent by weight			
Total protein	17.0 ± 6.3	18.6 ± 6.0	19.1 ± 0.9	12.3 ± 1.0[a]
Total lipid	83.1 ± 10.2	82.4 ± 5.3	80.9 ± 0.9	87.6 ± 2.2[a]
Triglycerides	31.5 ± 3.2	23.5 ± 1.4[a]	59.9 ± 1.7	51.0 ± 0.9[a]
Esterified cholesterol	27.2 ± 1.8	40.3 ± 2.3[a]	9.9 ± 0.9	22.1 ± 1.2[a]
Unesterified cholesterol	8.9 ± 2.9	7.4 ± 2.4	2.6 ± 0.8	2.8 ± 0.8
Phospholipid	15.6 ± 2.6	11.2 ± 1.1	8.7 ± 2.2	11.7 ± 2.3

Values are mean ± SE for 4 monkeys per group. (hypo = hypocholesterolemic, 113 ± 6 mg/dl; hyper = hypercholesterolemic, 273 ± 15 mg/dl).

[a]Differs significantly from hypocholesterolemic animals ($P < 0.05$).

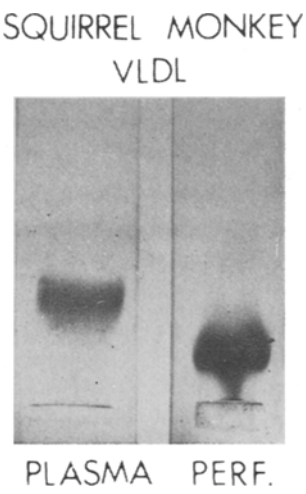

FIG. 2. A representative electrophoretogram of plasma and perfusate VLDL from a hypercholesterolemic monkey indicates a slower mobility of the perfusate VLDL. Differences in migration were not observed as a function of plasma cholesterol levels.

compared to that found in humans (34) and several experimental animals (35,36). An even greater enrichment (40% total lipoprotein) was observed in the hypercholesterolemic monkeys fed saturated fat, similar to findings in coconut oil-fed rabbits (5) and cholesterol-fed rabbits (4), guinea pigs (2,3), rats (1) and New and Old World monkeys (7-9). A less marked enrichment occurs in VLDL of rhesus fed coconut oil (37) and is minimal in coconut oil-fed cebus (38); the coconut oil-fed cebus is remarkable for its resistance to atherosclerosis compared to the squirrel monkey (39). These observations underscore the potential of the saturated fat component in any diet-induced enrichment of the cholesteryl ester fraction of VLDL and raises questions concerning their origin.

Circulating cholesteryl esters of VLDL are thought to originate from 3 sources: as a component of nascent hepatic VLDL, from plasma via the LCAT enzyme, and from intestinally-derived chylomicron remnants. These chylomicron remnants are probably a major source of increased plasma VLDL cholesteryl ester observed in cholesterol-fed rabbits (4). On the other hand, in the hypercholesterolemic rat (12) and guinea pig (3), secretion of cholesteryl ester-rich lipoproteins by isolated perfused livers suggests a major contribution by the liver for the esterified cholesterol of plasma VLDL. Studies in humans (40,41) indicate that a significant portion of the cholesteryl esters of plasma VLDL are derived from the transfer of esters formed in HDL by the action of LCAT.

The Portman et al. studies (14), which demonstrated a substitution of cholesteryl ester for the triacylglycerol of VLDL during alimentation, implicated an intestinal orgin for this cholesteryl ester-enriched lipoprotein. On the other hand, nascent hepatic VLDL obtained after Triton blockade in fasting squirrel monkeys were also cholesteryl ester-enriched (13), again pointing to the liver as a site of origin. The true source of these particles could not be ascertained from those studies, since plasma, obtained after Triton blockade, not only contains newly secreted hepatic VLDL but also VLDL of undefined origin in circulation at the time of tritonization. In addition, since Triton blockade also inhibits LCAT activity, an accurate indication of the extent of transfer of cholesteryl ester from HDL to VLDL could not be made.

TABLE II

Secretion of VLDL Protein and Lipids by Perfused Livers from Hypo- or Hypercholesterolemic Squirrel Monkeys

VLDL Component	Perfusate VLDL	
	Hypocholesterolemic	Hypercholesterolemic
	μg/g liver/3 hr	
Total protein	125 ± 6	124 ± 12
Total lipid	532 ± 13	881 ± 24[a]
Triglycerides	392 ± 26	513 ± 38[a]
Esterified cholesterol	65 ± 12	222 ± 19[a]
Unesterified cholesterol	17 ± 6	28 ± 9
Phospholipid	56 ± 12	118 ± 31[a]
Total VLDL lipoprotein	656 ± 23	1005 ± 34[a]

Mean ± SE for 4 monkeys per group.

[a]Differs significantly from hypocholesterolemic animals ($P < 0.05$).

In this study, the liver perfusion system enabled us to assess hepatic capability for secretion of cholesteryl ester-rich VLDL independent of extra-hepatic sources of these particles. Although the livers from hypercholesterolemic monkeys secreted particles containing relatively more cholesteryl ester (22% total lipoprotein) than VLDL secreted by livers of hypocholesterolemic animals (10% total lipoprotein), the particles were less enriched than VLDL circulating in plasma. Thus, these data support the in vivo Triton studies of Illingworth et al. (13) and also the in vitro liver perfusion studies in rats (18,19) and guinea pigs (3) which would suggest that at least part of the enrichment results from hepatic secretion of a cholesteryl ester-rich VLDL.

An apparent difference was found between the cholesteryl ester-rich lipoproteins secreted by the isolated perfused livers of hypercholesterolemic primates and rats. Although perfused livers of hypercholesterolemic squirrel monkeys failed to secrete increased amounts of LDL (unpublished results), perfused livers of hypercholesterolemic rats secrete cholesteryl ester-rich LDL which may contribute directly to plasma LDL (12). This apparently is not true in squirrel monkeys where the LDL is presumably derived from cholesteryl ester-rich VLDL (30).

The third objective of this study was to determine whether the livers of hypercholesterolemic monkeys would secrete more or less VLDL than those from hypocholesterolemic animals. A 300% increase in total VLDL output was associated with hypercholesterolemia, in agreement with the greater VLDL secretion noted from perfused livers of hypercholesterolemic rats (18,19), gerbils (20) and guinea pigs (3). These results are the opposite of our own (15,16) and other (14,17) investigations which demonstrate that triacylglycerol or VLDL secretion determined in vivo after Triton blockade is reduced in hypercholesterolemic animals. The explanation for this discrepancy remains obscure, but it seems plausible to hypothesize that serum factors present during in vivo measurements and absent in the in vitro liver perfusion systems may regulate lipoprotein secretion.

The enhanced VLDL-lipid secretion by the perfused livers of hypercholesterolemic monkeys contrasted with the comparable VLDL-protein secretion by the 2 groups of monkeys. This suggests that the hypercholesterolemic monkey liver secreted an equal number of larger particles. Similar findings have been reported for rat livers perfused with various fatty acids, i.e., VLDL triglyceride secretion and VLDL lipid composition varied, but total hepatic protein secretion was the same (42).

The mechanisms controlling in vitro VLDL particle secretion and the amount and kind of lipid it will transport remain to be elucidated. However, it has been suggested that enhanced VLDL secretion by perfused livers associated with such things as cholesterol feeding (18,19), saturated fat intake (20) or Triton-induced hyperlipemia (43) may be in response to a demand for transport of newly synthesized hepatic triglyceride (44). Although hepatic lipid concentration and synthesis could not be measured prior to perfusion of these monkey livers, liver slices and mitochondrial supernatants from hypercholesterolemic monkeys (21) and gerbils (20) fed coconut oil incorporate more lipid precursors into hepatic triacylglycerol or perfusate VLDL-lipid than normocholesterolemic animals fed unsaturated fat.

In conclusion, this investigation has demonstrated that (a) saturated fat-induced hypercholesterolemia in squirrel monkeys results in plasma VLDL enriched in cholesteryl ester, (b) perfused livers can secrete cholesteryl ester-rich nascent VLDL and (c) hypercholesterolemia results in enhanced VLDL production by the isolated perfused liver.

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Inhibition of Rat Hepatic Sterol Formation from Squalene by Plasma Lipoproteins

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ABSTRACT

The conversion of ^{3}H-squalene to sterols by rat liver microsomes and cytosol was inhibited by individual rat and human plasma lipoproteins at various concentrations. This inhibition was also observed with added human high density apolipoprotein, but triglycerides, cholesterol or cholesteryl esters had no inhibitory effects. Lipoproteins and apo high density lipoprotein (HDL) were demonstrated to bind ^{3}H-squalene in vitro. The binding of ^{3}H-squalene by apo HDL could be reversed by increasing concentration of liver cytosol containing sterol carrier protein$_1$.

INTRODUCTION

The lack of correlation between synthesis of cholesterol and activity of 3-hydroxy-3-methyl glutaryl CoA reductase in a variety of cultured cells and in rats treated with ML-236B (1-3) indicates that regulation of cholesterol biosynthesis at points beyond mevalonic acid may be of physiological importance. Regulation of cholesterol biosynthesis in the liver at steps beyond mevalonic acid has been suggested by several investigators. From studies on the effects of cholesterol feeding on hepatic synthesis of cholesterol and squalene in vivo, Rao and Olsen (4) concluded that there was possibly a control point for the biosynthesis of cholesterol at the cyclization of squalene. Burns et al. (5) incubated human peripheral leukocytes with ^{14}C-acetate and found that most of the radioactivity was incorporated into lanosterol, squalene and an unidentified sterol but not into cholesterol, suggesting that in human leukocytes rate-limiting steps exist beyond the 3-hydroxy-3-methyl glutaryl CoA reductase reaction. Onajobi and Boyd (6) showed that rat serum contained a protein termed "squalene sequestering protein," which inhibited the conversion of mevalonate to cholesterol. They showed that this protein was very similar to apo high density lipoproteins (HDL) and that it bound squalene irreversibly, suggesting a novel mode of control of cholesterol biosynthesis. In addition, our finding (7) that a hepatic cytosolic protein, sterol carrier protein$_1$ (SCP_1), acts specifically at the oxidation and cyclization of squalene by microsomes indicates that this step is an important point at which cholesterol biosynthesis is regulated. This work deals with a probable regulatory role of lipoproteins at this step.

MATERIALS AND METHODS

^{3}H-Squalene was prepared biosynthetically from DL-[5-^{3}H]-mevalonic (New England Nuclear, specific activity 1.0 Ci/mmol) by anaerobic incubation with a 20,000 × g supernatant of rat liver homogenate in a manner similar to that described by Tchen (8). The squalene was purified by passing the radioactive mixture through a 7-ml silicic acid (Unisil/Supercel 5:1) column in benzene/carbon tetrachloride (1:9) and then eluting with the same solvent mixture. This column procedure was repeated once more to obtain pure squalene. Its specific activity (sp act) was determined by liquid scintillation counting and colorimetric determination of squalene (9). Male Sprague Dawley rats (200-300 g) were used for preparation of liver microsomes and liver 105,000 × g supernatant (S_{105}). Livers were homogenized in 3 times their vol of buffer (0.02 M potassium phosphate buffer, pH 7.4, containing 10^{-4} EDTA) with a Polytron homogenizer. From this homogenate, the S_{105} and microsomes were prepared as previously described (7). The microsomes were washed once by suspending them in buffer equivalent to the initial vol of homogenate and then rehomogenizing with the Polytron. After centrifugation at 105,000 × g for 1 hr, the pellet was suspended in buffer to give a protein concentration of 10 mg/ml. SCP_1 was assayed in a reaction mixture of 2.0 ml buffer, microsomes (2 mg), NADPH (91.2 mM), NAD^+ (3 mM), 100 μg phosphatidylserine (prepared by sonicating 10 mg in 10 ml buffer at room temperature for 5 min), FAD (0.1 mM), ^{3}H-squalene (20,000 cpm, 1.0 nmol) in dioxane/propylene glycol (2:1) and S_{105} as a source of SCP_1. The incubations were carried out for 2 hr at 37 C in a Dubnoff shaker under oxygen. The remaining steps of the procedure were the same as described by Scallen et al. (10).

Lipoproteins were isolated from the rat plasmas by centrifugation according to the Havel et al. method (11). The serum was

brought to 1 mM EDTA and centrifuged at 105,000 × g for 18 hr, after which the top floating layer of very low density lipoprotein (VLDL) was removed. Sodium chloride was added to the infranatant to adjust the solvent density to 1.063. After centrifuging at 105,000 × g for 22 hr, the top layer of low density lipoprotein (LDL) was carefully removed. The infranatant was then adjusted to a solvent density of 1.21 by adding sodium bromide and centrifuged at 105,000 × g for 36 hr. This time the floating layer contained the HDL. The lipoproteins were dialyzed against 0.02 M potassium phosphate buffer (pH 7.4).

HDL was delipidated by a modification of the Shore and Shore procedure (12). The delipidation was done at 4 C in 10-ml graduated centrifuge tubes equipped with teflon-lined screw caps. Five ml of HDL in phosphate buffer, containing 4 mg HDL protein/ml, was placed in each tube and an equal vol of diethyl ether/ethanol (1:1, v/v) was injected rapidly into the solution with a syringe. The tubes were immediately rotated on a Multipurpose Rotator for 10-15 min to facilitate the extraction. The extraction mixture aws then centrifuged for 2 min to form 2 distinct phases. The lipid-containing upper phase was removed by aspiration and replaced by 1.5 ml of ether/ethanol (3:1 v/v), which was vigorously injected as before. The tubes were rotated and centrifuged, and the upper phase was removed and discarded. This procedure was repeated until the vol of the aqueous phase was reduced from ca. 8.5 ml to 5 ml. A total of 6-7 extractions with ca. 1.5-ml aliquots of the ether-ethanol mixture was required. After delipidation, the aqueous phase containing apo HDL was dialyzed against 0.1 M Tris buffer, pH 8.0, containing 0.1% EDTA to remove the organic solvent.

For the binding studies, 50,000 cpm (5 nmol) ^{3}H-squalene was incubated with 1 mg of each lipoprotein fraction for 15 min under nitrogen at 37 C; the mixture was then passed through a 10-ml Sephadex G-10 column. The column was eluted with the same buffer and 1.0-ml fractions collected. Radioactivities in each fraction were determined by liquid scintillation counting and protein concentration determined by absorption at 280 nm; afterwards, the percentage binding of squalene was calculated. The unbound ^{3}H-squalene remained on the column.

RESULTS

We investigated several proteins which could possibly bind squalene and act as competitive inhibitors of SCP_1. The lipoproteins that were isolated from liver S_{105} by flotation after centrifugation for 72 hr at 105,000 × g in a solvent density of 1.21 had the capacity to bind squalene. Subsequently, we isolated the VLDL, LDL and HDL by centrifugation from both rat plasma and human plasma (11). Each type of lipoprotein, when incubated with ^{3}H-squalene and eluted from a Sephadex G-10 column, showed a squalene binding of 3-3.5 nmol/mg protein.

Figures 1 and 2 show the inhibition by rat and human lipoproteins of the stimulatory effect of cytosol containing SCP_1 on microsomal conversion of squalene to sterols. This inhibition was not specific to any single type of lipoprotein. The inhibition was fully reversible by increasing the substrate concentration 10- to 20-fold. At higher concentrations of the lipoproteins, the activity of microsomal enzymes was suppressed to lower than control activity of microsomes without added cytosol, suggesting that some residual SCP_1 attached to microsomes is being inhibited. Triolein (1.0 mM), cholesterol (1.0 mM), or cholesteryl oleate (1.0 mM), added in 10 μl dioxane, propylene glycol (2:1) or sonicated triolein in buffer did not inhibit SCP_1 activation.

Cytosolic SCP_1 activation was also inhibited by human apo HDL when apo HDL was added into the reaction mixture at different concentrations (Table I). The inhibition by apo HDL could be reversed by adding higher concentrations of cytosol (Table II). In this experiment, the ^{3}H-squalene was used as squalene-HDL complex. Cytosol was still able to reverse the inhibition.

DISCUSSION

Squalene is the first water insoluble intermediate in the biosynthesis of cholesterol, and it is also the first precursor which requires SCP_1 in addition to microsomal enzymes in order to be converted into sterols (7). Squalene has been shown to bind to VLDL (13), LDL (14) and HDL (6) as well as to SCP (15). It was therefore important to determine the effects of these lipoproteins and their protein and lipid moieties, since all are synthesized in the liver and might compete there for binding of squalene. We found that all 3 types of lipoproteins and apo HDL bind squalene and that these were all inhibitory to activation of the microsomal enzymes by SCP_1. The crude preparation of S_{105} used here as a source of SCP_1 does contain proteins that float in the ultracentrifuge at a solvent density of 1.21 capable of binding radioactive squalene, but the concentration of these proteins in the actual reaction mixture in

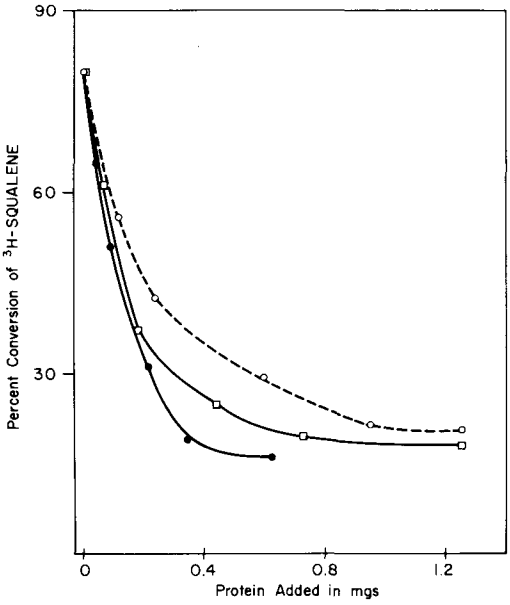

FIG. 1. Inhibition of rat liver SCP_1 activity by rat plasma lipoproteins: the 2-ml incubation mixtures contained 3.5 mg S_{105} proteins, different concentrations of rat HDL (○----○); of VLDL (□—□); or LDL (●----●) and other contents at concentrations as described in Methods.

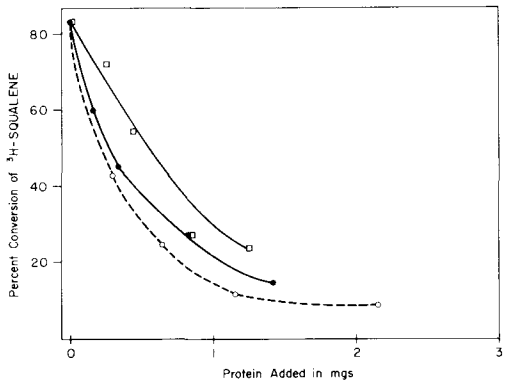

FIG. 2. Inhibition of rat liver SCP_1 activity by human plasma lipoproteins: the 2-ml incubation mixture contained 3.5 mg rat liver S_{105} proteins, different concentrations of human HDL (○----○); or VLDL (□—□); or LDL (●----●) and other contents at concentrations as described in Methods.

Figures 1 and 2 is far less compared to the added plasma lipoprotein inhibitors. Onajobi and Boyd (6) observed that when rat or human serum was added to an incubation mixture with all the cofactors, including a 19,000 × g rat liver supernatant and ^{14}C-mevalonic acid to synthesize cholesterol, incorporation of mevalonic acid into cholesterol was decreased and there was accumulation of squalene. Subsequently, they showed that the factor responsible for this squalene accumulation was very similar and possibly identical to apo HDL. Goodman observed (13) that almost all the squalene in normal human plasma is bound to VLDL. Lewis (14) found that in subjects who consumed a meal containing 1.5 mg of squalene there was an increase in the squalene content of plasma LDL after 3 hr. All of these studies provide evidence for the binding of squalene by lipoproteins. Our current studies show that all these lipoproteins and apo HDL inhibit SCP_1 in the liver cytosol. The recent characterization of a lipoprotein retinyl ester complex by Heller (16) from rat liver cytosol suggests a possible physiological importance to our finding. Prior to this finding, major lipoproteins had been demonstrated only in the Golgi apparatus (17-19). We found that retinol added as liposomes in phosphatidylcholine inhibited the

TABLE I

Inhibition of Cytosolic SCP_1 by apo HDL

Concentration of apo HDL μg/ml reaction mixture	Percent conversion of squalene to sterols
None	44.0
6.0	40.0
12.0	35.0
60.0	23.0

Reaction mixture (2.0 ml) contained 3.5 mg S_{105} protein, different concentrations of apo HDL as above and other constituents in concentrations as given in Methods.

TABLE II

Reversal of apo HDL Inhibition by Increased Cytosol Concentration

Concentration of S_{105} protein (mg/ml)	Percent conversion
None	18.0
1.7	54.0
3.5	74.0
7.0	84.0

^{3}H-Squalene (0.2 μCi) was incubated with 250 μg of apo HDL at 37 C under N_2 for 15 min and was applied to a Sephadex G-10 column. One-ml fractions were collected and the fractions with radioactivity were pooled. The 2-ml incubation mixtures contained 2 mg microsomes, 0.5 ml of the above pooled fractions, different concentrations of S_{105} as above and other constituents at concentrations as described in Methods.

conversion of squalene to sterol by rat liver microsomes and cytosol (unpublished observations). A recent report by Hashimoto and Dayton (20) showing that plasma lipoproteins from normal and hypercholesteremic rabbit serum stimulate cholesterol esterification in hepatic microsomes supports the concept that there may be some interaction between lipoproteins and the liver microsomes.

The inhibition of SCP_1 by lipoprotein is apparently competitive, since this inhibition could be reversed by increasing squalene concentrations. The inhibition does not result from any nonspecific sequestering of squalene by lipoproteins because the apo-HDL-bound ^{3}H-squalene becomes a substrate in the presence of higher concentrations of SCP_1. The inhibitory activity of the lipoproteins apparently results from their apo protein portion, since the lipid moieties such as phospholipid, triolein, cholesterol or cholesteryl ester are not inhibitory whereas apo HDL is inhibitory.

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Composition and Biosynthesis of Fatty Acids in *Pyramimonas grossii*

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ABSTRACT

The green alga *Pyramimonas grossii* orginating in the coastal waters of the Atlantic Ocean Argentina was subcultured until a monoalgal culture was obtained. The fatty acid composition of the alga grown in a mineral medium at 12 C was determined by gas liquid chromatography (GLC) on 2 columns. The major fatty acids were oleic, linoleic, palmitic and α-linolenic acids, but the 20-carbon polyunsaturated acids, 20:4ω6 and 20:5ω3, respectively, belonging to the linoleic and α-linolenic series, were also found. Incubation with [^{14}C] oleate, [^{14}C] acetate, [^{14}C] linoleate and [^{14}C] α-linolenate suggests that linoleate is not directly converted to α-linolenate. [^{14}C] Acetate was easily converted to palmitic, palmitoleic and oleic acids. However, after 48 hr of incubation, only traces of radioactivity were detected in linoleic acid and no label was found in α-linolenic acid.

INTRODUCTION

It has been established that the main fatty acids present in marine planktonic green algae belonging to the classes Chlorophyceae and Prasinophyceae are 16:4ω3, 18:3ω3 and 16:0, with smaller quantities of the acids 18:2ω6, 18:3ω6, 16:3ω6 and 16:3ω3 (1). In addition, it is reported that some species belonging to the genera *Dunaliella* and *Heteromastix* also contain significant quantities of 20:5ω3, 18:4ω3 and 22:5ω3 acids (2). The presence of smaller quantities of odd carbon number saturated acids and the corresponding anteiso acids were also reported in a freshwater Chlorophyte (3).

Two different routes have been proposed for the synthesis of α-linolenic acid in plants. In one pathway, it would be produced by the oxidative desaturation of linoleic acid in the ω3-bond position of the chain (4). The other route was reported by Kannangara et al. for the green alga *Chlorella pyrenoidosa* (5). These authors suggest the presence of an enzymatic system capable of desaturating dodecanoic acid to dodecatrienoic acid which is then elongated to α-linolenic acid.

We report the fatty acid composition of the unicellular Chlorophyte *Pyramimonas grossii* isolated from coastal waters of Argentina and the results of a preliminary study on fatty acid biosynthesis in this species.

EXPERIMENTAL PROCEDURES

Microorganisms

P. Grossii Parke (6) was isolated from a coastal sea water sample (San Antonio Oeste Bay, Río Negro, 42°44′ S-64° 54′W, Argentina). The sample volume of 300 ml was enriched with a mixture of potassium phosphate and sodium nitrate according to Castellvi's method (7). After 1 week, *P. grossii* became dominant and a monoalgal culture was obtained by successive dilutions.

Cultures were maintained in a lipid-free liquid medium, which consisted of (mmol/ℓ): $NaNO_3$ (1), NaH_2PO_4 (1×10^{-1}), $NaSiO_2$ (2×10^{-1}), Na_2 EDTA (3×10^{-2}), Tris (5), NaCl (400, $MgSO_4$ (20), $MgCl_2$ (10), KCl (10), $NaCO_3H$ (2), $FeCl_3$ (2×10^{-3}), H_3BO_3 (2×10^{-1}), $MnCl_2$ (7×10^{-3}), $ZnCl_2$ (8×10^{-4}), $COCl_2$ (2×10^{-5}), $CuCl_2$ (2×10^{-7}), Vit. B_{12} (10 μg/ℓ), and distilled water (1,000 ml). The cultures were continuously illuminated with a 15-watt tungsten lamp at 30 cm from the culture surface.

Prior to incubation with labeled sustances, the cells were grown in 1-ℓ flasks with 400 ml of the medium, maintained in an incubator at 12 C ± 0.5 with continuous bubbling of sterile air at ca. 400 ml/min. When cultures reached half of the logarithmic growth phase (between 1.5 and 2.0×10^6 cells/ml), cells were either separated for fatty acid analysis or incubated with radioactive substances.

No contamination of cell cultures with bacteria could be detected by microscopic examination and only a few colonies appeared after culturing in the same medium with 3% agar for 9 days. Therefore, as bacterial contamination was negligible, the labeling products were ascribed specifically to the alga.

Incubation with Labeled Substrates

[1-^{14}C] Sodium acetate (61 mCi/mmol), [1-^{14}C] oleic acid (62 mCi/mmol), [1-^{14}C]-

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linoleic acid (61 mCi/mmol) and [1-^{14}C]α-linolenic acid (58 mCi/mmol) of 98% purity were purchased from Radiochemical Centre, Amersham, England for use as radioactive tracers.

Algal cultures were incubated with 2 μmol sodium acetate (2 μCi) for 48 hr. One μmol (1 μCi) of each of the other precursors was added to cultures as ammonium salt and incubated for 24 hr under the conditions already described.

Some incubations with [1-^{14}C]sodium acetate and [1-^{14}C]ammonium oleate were carried out in the presence of 20 μmol of nonradioactive linoleic acid. After incubation, the cells were separated by centrifugation at 2,500 x g for 10 min and washed with small amounts of fresh culture medium.

Fatty Acid Separation

Lipids and pigments were extracted from whole cells by the Folch et al. procedure (8). However, the yield of fatty acids was less than the amount obtained by a direct saponification of cells. Therefore, cells were saponified with 10% KOH in ethanol. Nonsaponifiable lipids were extracted with petroleum ether, radioactivity counted and then discarded. The solution was acidified with HCl and free fatty acids were extracted with petroleum ether. They were immediately converted to the methyl esters by heating with HCl-methanol.

The radioactivity of the different fractions was measured by dissolving the samples in Bray's scintillation liquid (9) and counting them in a Packard instrument.

Fatty Acid Analysis

Total lipid fatty acid composition of the alga was determined by gas liquid chromatography (GLC) in a Packard 420 apparatus with hydrogen flame detector. Glass columns of 180 cm x 0.4 cm id packed with 15% EGSS-X on Chromosorb WHP (80-100 mesh) at 180 C and 15% EGSS-Y on Chromosorb W aw (80-100 mesh) at 190 C were used. A flow rate of 60 ml/min of nitrogen as gas carrier was used. Quantitation was performed by triangulation.

Chromatographic peaks obtained from both columns were tentatively identified by comparison of their relative retention times (rrt 18:0) with standards and checked by determination of their equivalent chain length values (ECL) (10). The number of carbons in the fatty acids was determined by hydrogenation (11) and GLC of the methyl esters of the saturated acids thus formed.

The number of double bonds was determined in some cases by separating the methyl esters by TLC-$AgNO_3$ (10%) and developing twice in hexane/diethyl ether/acetic acid (94:4:2) (12). Each fraction was reanalyzed by GLC.

Radioactive fatty acid analyses were carried out by gas liquid radiochromatography in a Model 893 Packard apparatus equipped with a proportional counter using 15% DEGS on Chromosorb W AW (80-100 mesh). The relative radioactivity in the different fatty acid peaks was determined by measuring the area of the counter output peaks by triangulation (13).

RESULTS AND DISCUSSION

The total fatty acid composition of *P. grossii* is shown in Table I. Two GLC columns of different liquid phase polarities were used to help in the identification of some peaks. Only saturated fatty acids of 12, 14, 16, 18 and 20 carbons were detected when the total fatty acid methyl esters were hydrogenated and rechromatographed.

Fractionation of the fatty acid methyl esters by TLC-$AgNO_3$ revealed the presence of saturated esters and unsaturated compounds of 1, 2, 3, 4 and 5 double bonds. Each fraction was analyzed again by GLC to confirm the

TABLE I

Fatty Acid Composition of *P. grossii*

Fatty acids	ECL[a]	Composition[b] (wt %)	
12:0	12.00	2.0	(0.2)
14:0	14.00	0.9	(0.0)
16:0	16.00	14.3	(0.6)
16:1ω7 (?)	16.63	4.7	(0.3)
16:2	17.45	4.8	(0.2)
16:3ω6	18.00	10.0[c]	(0.4)
18:0	18.00		
16:3ω3	18.52	21.4[d]	(0.9)
18:1	18.52		
16:4ω3	19.05	7.4	(0.2)
18:2ω6	19.42	17.1	(0.4)
18:3ω6	20.00	1.0	(0.1)
18:3ω3	20.42	10.6[e]	(0.5)
18:4ω3	21.06	2.2	(0.1)
20:4ω6	22.39	1.2	(0.1)
20:5ω3	23.47	1.4	(0.1)

[a]Equivalent chain length calculated from retention times measured on EGSS-X at 180 C.

[b]Results are the mean of 3 determinations ± extreme deviations of the mean, in brackets.

[c]The 16:3ω6 acid amounts to ca. 6% of total fatty acids.

[d]The 16:3ω3 acid amounts to ca. 1% of total fatty acids.

[e]May include some 20:1 acid.

TABLE II

Radioactivity Distribution in the Fatty Acids of *P. grossii* after Incubation with Labeled Substrates

Labeled fatty acids	Substrates					
	[1-^{14}C] Na Ac	[1-^{14}C] Na Ac + 18:2ω6	[1-^{14}C]18:1ω9	[1-^{14}C]18:1ω9 + 18:2ω6	[1-^{14}C]18:2ω6	[1-^{14}C]18:3ω3
16:0	29.7 (2.2)[a]	19.9 (1.1)[a]	8.7	2.2	9.8	5.2
16:1	34.5 (3.6)	15.6 (1.2)			10.4	3.4
16:3ω6 (?)	–	–	1.2	1.7	–	1.2
18:0	trace	1.6 (1.0)	trace	trace	–	18.8
18:1	35.8 (2.4)	62.9 (3.3)	84.7	83.4	11.3	10.6
18:2ω6	*trace*	trace	trace	trace	68.5	3.2
18:3ω3	–	–	2.8	5.9	–	57.6
20:1	–	–	2.6	6.8	–	–

[a]Results are the mean of 2 determinations ± extreme deviations of the mean, in brackets.

previously assigned identities of fatty acids.

Results show that the most abundant fatty acids are: octadecenoic, linoleic, palmitic and α-linolenic with values similar to those of *C. pyrenoidosa* as shown by Matucha et al. (3). Although *P. grossii* is a marine alga, it contains a high proportion of linoleic acid. However, a significant amount of linoleic acid was also reported in the marine unicellular green algae *Chlamydomonas sp.* (2) and *Dunaliella salina* (14). Nevertheless, the quantitative fatty acid composition of *P. grossii* is significantly different from the one described for unicellular green algae, including other members of the class *Prasinophyceae* (2). Other saturated acids of 12, 14 and 18 carbons accompanied palmitic acid. Mono-, di-, tri-, tetra- and pentaethylenic unsaturated acids belonging to the ω3 and ω6 families also were found (Table I). The abundance of 18:1 and 18:2ω6 acids in *P. grossii* and the presence of 18:4ω3, 20:4ω6 and 20:5ω3 show a similarity with the fatty acid composition of some nonplanktonic members of the Chlorophyceae group (15,16). However, we must take into account that the nitrogen content in the medium, light intensity, or growth cycle phase may affect the fatty acid composition in algae (17).

Fatty Acid Biosynthesis

The results obtained by gas liquid radiochromatography after incubating the alga with labeled substrates are shown in Table II. When cells were incubated for 48 hr with [1-^{14}C] sodium acetate, saponified and lipids extracted with petroleum ether, a part of the radioactivity (76%) remained in the aqueous phase. A significant amount of the label was found in the unsaponifiable material (19%) and only a small proportion in the fatty acids. Although significant decarboxylation of sodium acetate is possible, this suggests that sodium acetate dissolved in the medium is incorporated by the cell, but that it is not used preferentially for fatty acid synthesis but for other syntheses, such as for sterols. At any rate, incorporation in 16:0, 16:1, 18:0 and 18:1 acids and traces in 18:2 acid was demonstrated. The lack of incorporation into the other acids, especially 18:3, could be explained by a slower biosynthesis, compartmentalization of acetate usage, or alternative substrates.

When cells were incubated with [1-^{14}C] 18:2ω6 and [1-^{14}C] 18:3ω3 salts, labeling was only detected in the incubated precursors and the shorter chain acids, but not in the higher homologs. This suggests that these fatty acids were incorporated but were possibly broken down to acetyl-CoA which was then

used by the cell in the synthesis of new short chain fatty acids. This is the most likely interpretation since it is very difficult to accept either a biohydrogenation of 18:2 to 18:1, or 18:3 to 18:2, 18:1 and 18:0 or a chain shortening of 18:3 to 16:3. In addition, the results of Table II suggest that in *P. grossii*, $\Delta 6$ as well as $\omega 3$ desaturases and elongases are inactive in our experimental conditions or that the added substrates do not reach the corresponding enzymes. $\Delta 6$ Desaturase is considered a typical enzyme of animals and $\omega 3$ desaturase is typical of plants. However, activity of both enzymes has been reported in some unicellular photosynthetic, as well as heterotrophic organisms (18-21).

Therefore, these results suggest the existence of other mechanisms different from those of direct desaturation of 18:2$\omega 6$ and 18:3$\omega 3$ to 18:3$\omega 3$ and 18:4$\omega 3$ acids, respectively. A possible route for the synthesis of α-linolenic acid in *P. grossii* could be the one proposed by Stumpf et al. for spinach chloroplasts, *C. pyrenoidosa* and *Candida bogoriensis* (5,22,23) starting from acids of low molecular weight.

After incubating the alga with [1-^{14}C] 18:1$\omega 9$, labeling was observed in some acids of lower molecular weight as well as in 18:2$\omega 6$, 18:3$\omega 3$ and 20:1. It seems likely that 20:1 is synthesized by chain elongation of the 18:1 precursor. With regard to the polyunsaturates, however, 2 possible synthetic pathways must be considered. One involves degradation of 18:1$\omega 9$ to acetate and de novo synthesis of products; the second is the conversion of 18:1$\omega 9$ to 18:2$\omega 6$ by a $\omega 6$ desaturase and thence to 18:3$\omega 3$ by a $\omega 3$ desaturase. However, as already discussed, [1-^{14}C] 18:2$\omega 6$, when directly supplied to the alga, was not converted to 18:3$\omega 3$. Therefore, the evidence points strongly toward degradation of 18:1$\omega 9$ to acetate and de novo synthesis of 16:3$\omega 3$, which is then elongated to 18:3$\omega 3$, by Stumpf's pathway. This de novo pathway is supported by the observation of labeled short chain fatty acids as additional reaction products.

The conversion of [^{14}C]18:1 to 18:3 acid is facilitated by adding unlabeled linoleic acid to the medium (Table II, column 4), whereas the conversion to palmitic acid is decreased. In addition, when unlabeled linoleic acid is added to the culture medium, the incorporation of [^{14}C]acetate into fatty acids is also affected markedly (Table II, column 2). Linoleic acid facilitates the incorporation of acetate into 18:1 but incorporation into palmitic and 16:1 acids is reduced. Thus, *P. grossii* is able to synthesize saturated and unsaturated fatty acids, resulting in a composition rich in palmitic, stearic, oleic, linoleic and α-linolenic acids, but the type and amount of acids synthesized would be controlled by the concentration of linoleic acid and also probably by other fatty acids in the medium.

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Oxidation of 3-Oxygenated Δ^4- and Δ^5-C_{27} Steroids by Soybean Lipoxygenase and Rat Liver Microsomes

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ABSTRACT

The formation of dioxygenated metabolites of cholesterol, epicholesterol (5-cholesten-3α-ol) 4-cholesten-3β-ol, 4-cholesten-3α-ol, 4-cholesten-3-one and 4-stigmasten-3-one was studied after incubations with soybean lipoxygenase and linoleic acid. From cholesterol and epicholesterol were formed the 7α-hydroxy-, 7α-hydroperoxy-, 7β-hydroxy-, 7β-hydroperoxy-, 7-oxo and 5,6-epoxy-derivatives as well as 6β-hydroxy-4-cholesten-3-one. All Δ^4-steroids were hydroxylated in the 6α- and 6β-positions. The ratios between the yields of 6β- and 6α-hydroxylated metabolites varied between 3:1 and 2:1. Incubations with 4-cholesten-3α-ol and 4-cholesten-3β-ol also afforded the 4,5-epoxides of these steroids. The ratios between the yields of the 4β,5β- and 4α,5α-epoxides were ca. 4:1 for 4-cholesten 3β-ol and ca. 3:2 for 4-cholesten-3α-ol. With iron-supplemented microsomes from rat liver, the compounds formed were qualitatively and quantitatively the same as with soybean lipoxygenase, whereas with 18,000 × g rat liver supernatant fractions the yields of all products formed—except 7α-hydroxycholesterol and 6β-hydroxy-4-cholesten-3-one—were markedly decreased. The results indicate the presence of a rat liver microsomal 6β-hydroxylase which can use 4-cholesten-3-one as a substrate and extend previous findings of similarities between soybean lipoxygenase and a nonspecific lipoxygenase in rat liver microsomes.

NOMENCLATURE

Epicholesterol, 5-cholesten-3α-ol; 4-stigmasten-3-one, 24α$_F$-ethyl-4-cholesten-3-one; TMS ether, trimethylsilyl ether; t_R, retention time relative to that of 5α-cholestane.

INTRODUCTION

Incubation of 3β-hydroxy-5-ene-steroids with soybean lipoxygenase (E.C. 1.13.11.12, also called soybean lipoxidase) and linoleic acid have been performed by Johansson (1), Aringer and Eneroth (2) and Teng and Smith (3,4). Johansson found that cholesterol afforded the same pattern of products as under conditions stimulating rat liver microsomal NADPH-dependent lipid peroxidation. Among the products identified were the 7α-hydroxy-, 7β-hydroxy- and 7-oxo-derivatives. Aringer and Eneroth could demonstrate that, apart from the 7-oxygenated derivatives of cholesterol, the 5β,6β- and 5α,6α-epoxy-derivatives were formed. β-Sitosterol afforded the same pattern of products as cholesterol after incubation with soybean lipoxygenase and linoleic acid, as well as under conditions stimulating rat liver microsomal NADPH-dependent lipid peroxidation (2). Teng and Smith found that cholesterol incubated with soybean lipoxygenase (under conditions slightly different from those used by Johansson, Aringer and Eneroth) afforded the epimeric 7-hydroperoxides as the major products. Teng and Smith have also oxidized 4-cholesten-3β-ol and 4-cholesten-3-one with soybean lipoxygenase (5). The major product identified with this first substrate was 4-cholesten-3-one, whereas the predominating metabolites of 4-cholesten-3-one were epimeric 6-hydroperoxy- and 6-hydroxy-derivatives as well as the 6-oxo-derivative.

During studies on the metabolism in rat liver subcellular fractions of isomeric 3-oxygenated C_{27}- and C_{29}-steroids with Δ^4- and Δ^5-configuration, we found that several different dioxygenated metabolites were formed (6,7). The yields of some of these metabolites were increased under conditions favoring nonspecific lipid peroxidation in rat liver microsomes. In order to investigate which dioxygenated metabolites can be formed by lipid peroxidation without the presence of tissue components, this study with soybean lipoxygenase was undertaken.

MATERIALS

Solvents

All solvents and reagents were analytical grade purchased from Merck AG, Darmstadt, West Germany, unless otherwise stated. They were used without further purification.

Substrates and Reference Steroids

All substrates were prepared and purified as described previously (6). 5α-Cholestane-3β,5-diol, 5β-cholestane-3β,5-diol, 4α,5-epoxy-5α-cholestan-3β-ol and 4β,5-epoxy-5β-cholestan-3β-ol were gifts from Prof. S. Bergström, Karolinska Institutet, S-104 01 Stockholm, Sweden. 4β,5-Epoxy-5β-cholestan-3-one and

4α,5-epoxy-5α-cholestan-3-one were obtained in 53 and 20% yields, respectively, from 4-cholesten-3-one which was oxidized with hydrogen peroxide and sodium hydroxide as described by Henbest and Jackson (8). The epoxides were purified by thin layer chromatography (TLC) in system 2 (see Table I). Reduction with sodium borohydride in dioxan-water (4:1, v/v) (11) afforded 4β,5-epoxy-5β-cholestan-3α-ol and 4β,5-epoxy-5β-cholestan-3β-ol in the proportions 2:1 from 4β,5-epoxy-5β-cholestan-3-one and 4α,5-epoxy-5α-cholestan-3α-ol and 4α,5-epoxy-5α-cholestan-3β-ol in the proportions 1:5 from 4α,5-epoxy-5α-cholestan-3-one. These compounds were acetylated and purified by TLC in system 2. After mild saponification (12), final purification was performed by TLC in system 1 (see Table I).

6β-Hydroperoxy-4-cholesten-3-one was purchased from Steraloids Inc., Pawling, NY. Sodium borohydride reduction in methanol of this compound afforded 4-cholestene-3α,6β-diol and 4-cholestene-3β,6β-diol (13). 6β-Hydroxy-4-cholesten-3-one was obtained in low yields by oxidation of 4-cholestene-3β,6β-diol with cholesterol oxidase as described by Richmond (14). The 6-hydroxy-Δ^4-steroids were purified in TLC system 1. 5-Cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol, 3β-hydroxy-5-cholesten-7-one, 5,6α-epoxy-5α-cholestan-3β-ol and 5,6β-epoxy-5β-cholestan-3β-ol were prepared and purified as described previously (2,15).

All reference steroids were analyzed by gas liquid chromatography-mass spectrometry (GLC-MS). The chromatographic properties of those compounds which we have not characterized previously (6) are presented in Table I, using the same conditions as in the earlier work.

Liquid Chromatography

Hydroxvalkylated, 55% substituted Sephadex LH-20 (140-170 mesh) was synthesized with a mixture of C_{11}-C_{14}-epoxides as described by Ellingboe et al. (16). The columns (0.75 cm^2 × 13 cm) were used in methanol/water/dichloroethane (95:5:25, v/v). Flow rate was 0.5 mL/cm^2 min at 24 C.

Thin Layer Chromatography

Unless otherwise stated, precoated Silica Gel 60 plates (Merck) were used in the solvent systems described in Table I or in the text.

TABLE I

Chromatographic Properties of Reference Steroids

Steroid analyzed	TLC[a] System 1	TLC[a] System 2	GLC[b]
5α-Cholestane-3β,5-diol	0.18		2.56
5β-Cholestane-3β,5-diol	0.50		3.34
4-Cholestene-3α,6β-diol	0.30		2.04
4-Cholestene-3β,6β-diol	0.15		2.38
6β-Hydroxy-4-cholesten-3-one	0.46		2.72
6β-Hydroperoxy-4-cholesten-3-one	0.55		
4α,5-Epoxy-5α-cholestan-3α-ol	0.57	0.45	2.70
4β,5-Epoxy-5β-cholestan-3α-ol	0.66	0.47	1.87
4α,5-Epoxy-5α-cholestan-3β-ol	0.55	0.52	2.62
4β,5-Epoxy-5β-cholestan-3β-ol	0.55	0.45	2.51
4α,5-Epoxy-5α-cholestan-3-one	0.91	0.62[c]	2.16[c]
4β,5-Epoxy-5β-cholestan-3-one	0.94	0.71[c]	1.93[c]

[a]The figures denote R_f-values. System 1: glass plates 20 × 20 cm, coated with Silica Gel G (Merck A.G., Darmstadt, West Germany). Solvent system, diethyl ether/cyclohexane (9:1, v/v). System 2: precoated Silica Gel 60 plates, 20 × 20 cm (Merck A.G.). Solvent system, benzene/ethyl acetate (15:1, v/v). The steroids were analyzed as their acetate derivatives in system 2.

[b]The figures denote retention times relative to that of 5α-cholestane (t_R-values). A Pye gas chromatograph (Model 104) equipped with a hydrogen flame ionization detector was used. The column contained Supelcoport 100-120 mesh coated with 3% SP-2100 (Supelco Inc., Bellefonte PA). The temperatures were: flash heater 270 C and column oven 255 C. Carrier gas was nitrogen and gas flow rate was 480 mL/cm^2/min. All compounds containing a hydroxyl group were analyzed as their TMS ethers. Except for 5β-cholestane-3β,5-diol, the TMS ethers were prepared as described by Makita and Wells (9). With this compound, the di-TMS ether had to be prepared with a trimethylbromosilane-containing reagent mixture as described previously (10). With the conditions used, all hydroxyl groups but no oxo groups were trimethylsilylated.

[c]Not derivatized.

Separated compounds were detected by iodine vapor or by radioautography (see following). Compounds were extracted from the gel with 5 mL of chloroform/methanol (4:1, v/v)/cm^2 of gel followed by 5 mL of methanol/cm^2 of gel. For identification purposes, the plates were sprayed with a solution of 70% H_2SO_4 saturated with $K_2Cr_2O_7$ and then heated at 120 C.

Gas Liquid Chromatography

See Table I, footnote b.

Gas Liquid Chromatography-Mass Spectrometry

This type of analysis was carried out with an LKB 9000 instrument equipped with a 1.5% SE-30 column (2 m × 3 mm) operated at 250 C. Helium was used as carrier gas. Flash heater: 270 C; molecular separator: 280 C; ion source: 290 C; energy of bombarding electrons: 22.5 eV.

Measurement of Radioactivity

A Packard Model 2009 liquid scintillation spectrometer was used. At least 2,000 counts above the background were counted. ^{14}C-Labeled spots on thin layer chromatograms were localized by exposing a sheet of Agfa Gevaert M3 for 1-4 days. GLC analysis with radioactivity detection (GLC-RD) was accomplished with a Packard Model 894 instrument. At least 2,000 dpm were injected.

EXPERIMENTAL PROCEDURES

Experiments with Soybean Lipoxygenase

Thirty mL of 0.1 M Tris-Cl buffer, pH 7.5 was kept at 30 C in a water bath for 5 min. The incubation flask was then vibrated and a stream of nitrogen was continously blown into the flask (15). Linoleic acid (12 mg), Sigma, grade III (see following), 500 μg of 4-^{14}C-labeled substrate in 1 mL of acetone, and 3 mg of Lipoxidase, Type I (E.C. 1.13.11.12), Sigma, in 250 μl of the Tris-Cl buffer, were added after 20, 30 and 40 sec, respectively. Prior to addition, the linoleic acid was converted into its ammonium salt by the addition of 0.25 ml of 0.019 M NH_4OH/mg of linoleic acid (1). The vibration under nitrogen was continued for 120 additional sec and then the flask was placed in the water bath. Shaking was initiated and a stream of air was blown into the flask for 30 sec. The incubation was then continued for an additional 30 min.

To terminate incubation, the reaction mixture was transferred into a separation funnel containing 60 mL of $CHCl_3$/MeOH (2:1, v/v). The incubation vessel was rinsed with another 60 mL of $CHCl_3$/MeOH (2:1, v/v) and 10 mL of 0.9% NaCl (w/v). The chloroform phase was collected and the water phase was extracted with 90 mL of $CHCl_3$/MeOH (2:1, v/v). The combined chloroform phases were taken to dryness and then purified by liquid chromatography (LC) (see Materials). Fractions were collected as follows: fraction I, 0-60% TCV; fraction II, 60-180% TCV; and fraction III, 180-400% TCV for all C_{27}-substrates. For 4-stigmasten-3-one, the fractions were collected somewhat differently, i.e., fraction I, 0-65% TCV; fraction II, 65-210% TCV; and fraction III, 210-450% TCV.

No attempts were made to identify metabolites from LC fraction I. Fraction II (which contained the dioxygenated steroids except the 3-oxo-4,5-epoxides) and fraction III (which contained the monooxygenated steroids and the 3-oxo-4,5-epoxides) were subjected to TLC followed by radioactivity detection (TLC-RD) in solvent system 1 (see Table I) or in benzene/ethyl acetate (7:1, v/v), respectively. The metabolites were extracted from the gel and the amount of radioactivity was determined. The TMS ethers were prepared (9) and the metabolites were analyzed by GLC-RD and GLC-MS. A metabolite was considered as identified according to the standard procedure when the TLC mobility, GLC mobility and the mass spectrum were identical to those of the authentic reference compound.

The mass spectra of all identified compounds, reference compounds and substrates can be made available through the World Health Organization (WHO) collection of mass spectral data for steroids, Dr. Jan Sjövall, Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden.

Experiments with Rat Liver Subcellular Preparations

Iron-supplemented rat liver microsomes were prepared from cholestyramine-treated rats (15) as described by Mitton et al. (17). Microsomes equivalent to 1 g wet liver suspended in 4.25 mL 154 mM KCl solution and 4.25 mL 0.1 M Tris-Cl buffer, pH 7.4, were used for incubations with 5-100 μg of 4-^{14}C-labeled substrate for 30 min at 37 C. The incubation buffer was fortified with NADPH, ADP and $FeSO_4$ to give a final concentration of 1.1 mM, 1 mM and 0.2 mM, respectively (17).

Supernatant fractions (18,000 × g) were prepared (from cholestyramine-treated rats) in a 0.25 M sucrose solution containing 0.01 M β-mercaptoethylamine and 0.001 M EDTA as

described previously (15). Incubations were performed at 37 C for 30 min with 5-100 μg of 4-^{14}C-labeled substrate and 10 μmol NADPH (15). Prior to the incubations, a 0.1 M potassium phosphate buffer, pH 7.0, containing 0.028 M nicotinamide and 0.01 M β-mercaptoethylamine was added so that each incubation flask contained 8.5 mL of a 1:1 (v/v) mixture of the sucrose solution and the phosphate buffer (15).

The metabolites from incubations with rat liver subcellular fractions were purified and identified exactly as just described for the products from the incubations with soybean lipoxygenase.

RESULTS

Products of Cholesterol

The yields of radioactivity from LC fractions I and II were 5 and 21%, respectively. The TLC-RD analysis of LC fraction II is illustrated in Figure 1. Compounds A1-A4 were identified as 5-cholestene-3β,7α-diol (A1), 5-cholestene-3β,7β-diol (A2), 3β-hydroxy-5-cholesten-7-one (A3) and a mixture of 5,6β-epoxy-5β-cholestan-3β-ol and 5,6α-epoxy-5α-cholestan-3β-ol in the proportions 4:1 (A4) as described previously (2).

The material eluted from positions A5 and A6 were each (after preparation of the TMS ethers) subjected to GLC-RD and GLC-MS analyses. Compounds with mobilities and mass spectra as 5-cholestene-3β-7α-diol, 6β-hydroxy-4-cholesten-3-one and 3β-hydroxy-5-cholesten-7-one were the main products primarily from position A5, whereas compounds with GLC motilities and mass spectra as 5-cholestene-3β,7β-diol and 3β-hydroxy-5-cholestene-7-one were the major products of preliminary identification from position A6. Small amounts of 5-cholestene-3β,7β-diol were also identified from position A5 and small amounts of 5-cholestene-3β,7α-diol were identified from position A6. In order to explain this discrepancy between the results of the TLC-RD analysis on one hand and the GLC-RD and GLC-MS analysis on the other hand, aliquots of the materials eluted from positions A5 and A6 were again (without preceding trimethylsilylation) subjected to TLC-RD analysis in system 1. The major part of the radioactivity was then located at the same position as authentic 5-cholestene-3β,7α-diol, 3β-hydroxy-5-cholesten-7-one and 6β-hydroxy-4-cholesten-3-one (compounds A5) or as 5-cholestene-3β,7β-diol and 3β-hydroxy-5-cholesten-7-one (compounds A6). Aliquots of compounds A5 and A6, respectively, were also reduced with sodium borohydride in methanol. Compound(s) A5 afforded 5-cholestene-3β,7α-diol, 5-cholestene-3β,7β-diol and 4-cholestene-3β,6β-diol as the main products. Reduction of compound(s) A6 yielded mainly 5-cholestene-3β,7β-diol and also minute amounts of 5-cholestene-3β,7α-diol. These results indicate the presence of 3β-hydroxy-5-cholesten-7α-hydroperoxide at position A5 and of 3β-hydroxy-5-cholesten-7β-hydroperoxide at position A6.

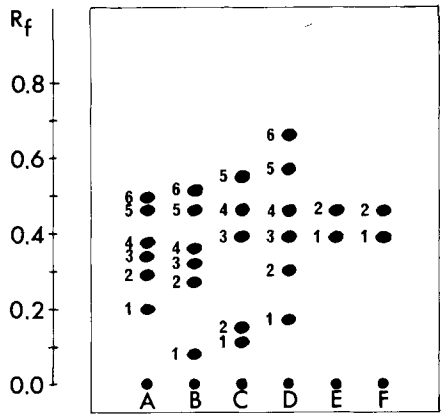

FIG. 1. Product pattern (TLC-RD) sketch after incubation of Δ^4- and Δ^5-steroids with soybean lipoxygenase. Solvent system: diethyl ether/cyclohexane (9:1, v/v). A-F represent incubations with: cholesterol (A), epicholesterol (B), 4-cholesten-3β-ol (C), 4-cholesten-3α-ol (D), 4-cholesten-3-one (E) and 4-stigmasten-3-one (F). Compounds A1-A4 were identified as 5-cholestene-3β-7α-diol (A1), 5-cholestene-3β,7β-diol (A2), 3β-hydroxy-5-cholesten-7-one (A3) and a mixture of 5,6β-epoxy-5β-cholestan-3β-ol and 5,6α-epoxy-5α-cholestan-3β-ol (A4). Compounds C3, D3, E1, and F1 were all found to be identical with 6α-hydroxy-4-cholesten-3-one, and compounds C4, D4, E2 and F2 were identical to 6β-hydroxy-4-cholesten-3-one. For the identities of the other compounds, see Results.

The yields of compounds from LC fraction II are presented in Table II. The yields of the 7-hydroperoxides have been calculated as the sum of the yields of 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol after sodium borohydride reduction of the material at position A5 (7α-hydroperoxide) or A6 (7β-hydroperoxide), respectively.

TLC-RD analysis of LC fraction III afforded one major radioactive spot. This radioactive compound was identified as cholesterol according to the standard procedure. The yield was 70%. Small amounts (0.2% yield) of 4-cholesten-3-one were also found in this LC fraction.

Products of Epicholesterol

The yields of radioactivity from LC fractions I and II were 5 and 27%, respectively. TLC-RD

TABLE II

Yields of Dioxygenated Products after Incubations of Δ^4- and Δ^5-Steroids with Soybean Lipoxygenase (%)[a]

Substrate	Metabolite formed	Soybean lipoxygenase	Iron-supplemented microsomes	18,000 × g supernate
5-Cholesten-3β-ol	5-Cholestene-3β,7α-diol	3.1	2.6	2.9
	5-Cholestene-3β,7β-diol	2.5	2.0	0.2
	3β-Hydroxy-5-cholesten-7-one	6.1	7.2	0.2
	5,6α-Epoxy-5α-cholestan-3β-ol	0.5	0.7	< 0.1
	5,6β-Epoxy-5β-cholestan-3β-ol	2.0	2.4	0.2
	6β-Hydroxy-4-cholesten-3-one	0.3	0.3	< 0.01
	3β-Hydroxy-5-cholesten-7α-hydroxyperoxide	0.3	0.3	< 0.01
	3β-Hydroxy-5-cholesten-7β-hydroxyperoxide	1.6	1.2	< 0.01
5-Cholesten-3α-ol	5-Cholestene-3α,7α-diol	2.3	3.4	0.2
	5-Cholestene-3α,7β-diol	2.4	2.9	0.3
	3α-Hydroxy-5-cholesten-7-one	5.6	16.3	0.3
	5,6-Epoxycholestan-3α-ol	2.5	8.0	0.2
	6β-Hydroxy-4-cholesten-3-one	0.3	0.2	< 0.01
	3α-Hydroxy-5-cholesten-7α-hydroxyperoxide[b]	< 0.1	0.2	< 0.01
	3α-Hydroxy-5-cholesten-7β-hydroxyperoxide[b]	0.3	0.4	< 0.01
4-Cholesten-3β-ol	4-Cholestene-3β,6α-diol	0.2	0.1	< 0.01
	4-Cholestene-3β,6β-diol	0.4	0.3	< 0.01
	6α-Hydroxy-4-cholesten-3-one	0.3	0.4	< 0.01
	6β-Hydroxy-4-cholesten-3-one	0.8	1.1	< 0.02
	4α,5-Epoxy-5α-cholestan-3β-ol	0.1	0.1	< 0.03[c]
	4β,5-Epoxy-5β-cholestan-3β-ol	0.4	0.5	
4-Cholesten-3α-ol	4-Cholestene-3α,6α-diol	0.2		< 0.03
	4-Cholestene-3α,6β-diol	0.5		< 0.05
	6α-Hydroxy-4-cholesten-3-one	0.1		< 0.03
	6β-Hydroxy-4-cholesten-3-one	0.2		0.3
	4α,5-Epoxy-5α-cholestan-3α-ol	0.7		0.2
	4β,5-Epoxy-5β-cholestan-3α-ol	1.1		0.5
4-Cholesten-3-one	6α-Hydroxy-4-cholesten-3-one	0.3	0.5	< 0.04
	6β-Hydroxy-4-cholesten-3-one	0.9	1.8	0.6
4-Stigmasten-3-one	6α-Hydroxy-4-stigmasten-3-one	0.4		< 0.03
	6β-Hydroxy-4-stigmasten-3-one	1.1		0.2

[a]Each figure represents the mean of 2-4 experiments. Incubations with iron-supplemented microsomes and 18,000 × g supernate were performed with 5 μg of substrate.

[b]Tentatively identified.

[c]Means the sum of yields of the 2 isomeric 4,5-epoxides.

analysis of LC fraction II revealed the metabolite pattern shown in Figure 1. Metabolites B1-B6 were each eluted from the gel and analyzed by GLC-RD and GLC-MS. Metabolite B1 was further purified by TLC-RD in the solvent system ethyl acetate/cyclohexane (6:1, v/v) yielding one single labeled spot with an R_f value of 0.21.

GLC-RD analyses of the isolated compounds B1 and B2 on a 3% SP-2100 column yielded only one labeled peak each (t_R = 2.17 and t_R = 2.15, respectively). Both compounds gave rise to a mass spectrum which was almost identical to that of 5-cholestene-3β,7α-diol (or 5-cholestene-3β,7β-diol) (15).

The TMS ethers of compounds B3 and B4 were eluted at t_R = 3.18 and t_R = 2.26, respectively, on the SP-2100 column. GLC-MS analysis yielded mass spectra which were identical with those of the TMS ethers of 3β-hydroxy-5-cholesten-7-one (15) and 5,6-epoxides of cholesterol (2), respectively. However, the t_R-values for 3β-hydroxy-5-cholesten-7-one, 5,6α-epoxy-5α-cholestan-3β-ol and 5,6β-epoxy-5β-cholestan-3β-ol were 3.79, 2.76 and 2.67, respectively. Compounds B3 and B4 were therefore identified as 3α-hydroxy-5-cholesten-7-one and 5,6-epoxycholestan-3α-ol, respectively.

Sodium borohydride reduction of 3α-hydroxy-5-cholesten-7-one in methanol yielded compounds B1 and B2 in the proportions 1:3. Incubation of compounds B1 and B2 with rat liver microsomes fortified with NAD as described previously (18) gave rise to compounds with the same chromatographic properties (TLC system 1 and GLC-RD) as 7α-hydroxy-4-cholesten-3-one (3% yield) and 7β-hydroxy-4-cholesten-3-one (6% yield), respectively. In parallel experiments, 5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol yielded 7α-hydroxy-4-cholesten-3-one (35% yield) and 7β-hydroxy-4-cholesten-3-one (2% yield), respectively. The combined results justified the identification of compound B1 as 5-cholestene-3α,7α-diol and compound B2 as 5-cholestene-3α,7β-diol.

The compounds eluted from positions B5 and B6 were reduced with sodium borohydride in methanol. The major products then identified according to the standard procedure were 4-cholestene-3β,6β-diol from position B5 and 5-cholestene-3α,7β-diol from position B6. From the material at position B5 small amounts of 5-cholestene-3α,7α-diol were also identified. These results indicate that the mixture at position B5 was composed of 6β-hydroxy-4-cholesten-3-one and smaller amounts of 3α-hydroxy-5-cholesten-7α-hydroperoxide and that the predominant compound at B6 was identical to 3α-hydroxy-5-cholesten-7β-hydroperoxide.

The yields of compounds from LC fraction II are presented in Table II. The yields of 6β-hydroxy-4-cholesten-3-one and of the tentatively identified 3α-hydroxy-5-cholesten-7α-hydroperoxide, and 3α-hydroxy-5-cholesten-7β-hydroperoxide are calculated as the yields of 4-cholestene-3β,6β-diol, 5-cholestene-3α,7α-diol and 5-cholestene-3α,7β-diol, respectively, after reduction with sodium borohydride.

TLC-RD analysis of LC fraction III yielded one major radioactive spot. This radioactive compound was identified as epicholesterol according to the standard procedure. The yield was 60%. Small amounts (0.7% yield) of 4-cholesten-3-one were also found in this LC fraction.

Products of 4-Cholesten-3β-ol

The yields of radioactivity from LC fractions I and II were 2 and 11%, respectively. The metabolite pattern after TLC-RD analysis of LC fraction II is shown in Figure 1. Compounds C2, C4 and C5 were identified as 4-cholestene-3β,6β-diol (C2), 6β-hydroxy-4-cholesten-3-one (C4), and 4β,5-epoxy-5β-cholestan-3β-ol (C5), according to the standard procedure.

GLC-RD analysis of the TMS ethers of compounds C1 and C3 yielded radioactive peaks at t_R = 2.77 (C1) and t_R = 3.63 (C3) compared to t_R = 2.38 and t_R = 2.72 for the TMS ethers of compounds C2 and C4. GLC-MS analyses of the TMS-ethers of compounds C1 and C3 gave rise to mass spectra with the same fragments as were present in the mass spectra of the di-TMS-ether of 4-cholestene-3β,6β-diol and the mono-TMS ether of 6β-hydroxy-4-cholesten-3-one, respectively. Thus, prominent peaks were seen in the high end of the mass spectrum at m/z 546 (M), 10%; 531, 6%; 517, 2%; 456, 100%; 441, 45%; 403, 79% for compound C1 and at m/z 472 (M), 44%; 457, 100%; 444, 9%; 416, 87%; 401, 12% for compound C3. However, for the di-TMS ether of 4-cholestene-3β,6β-diol, the relative intensities of these fragments were: m/z 546, 23%; 531, 33%; 517, 16%, 456, 72%; 441, 45%; 403, 100% and for the mono-TMS ether of 6β-hydroxy-4-cholesten-3-one: m/z 472, 40%; 457, 91%; 444, 10%; 416, 100%; 401, 10%, respectively. Sodium borohydride reduction in isopropanol of metabolite C3 afforded metabolite C1 as the major product. Compounds C1 and C3 were thus identified as 4-cholestene-3β, 6α-diol and 6α-hydroxy-4-cholesten-3-one, respectively.

The TMS ethers of 4α,5-epoxy-5α-cholestan-3β-ol and 4β,5-epoxy-5β-cholestan-3β-ol had the same R_f-values in TLC system 1 and similar

t_R-values on an SP-2100 column (see Table I). The mass spectra were almost identical. To examine whether compound(s) C5 (4β,5-epoxy-5β-cholestan-3β-ol) also contained small amounts of 4α,5-epoxy-5α-cholestan-3β-ol, the acetate derivatives were prepared and analyzed by TLC-RD in system 2 (see Table I). Radioactive spots were detected at R_f = 0.45 (70% yield) and R_f = 0.52 (15% yield). The radioactive material with the lower R_f-value was identified as (GLC-RD and GLC-MS) 3β-acetoxy-4β,5-epoxy-5β-cholestan. The radioactive material with the higher R_f-value had the same R_f-value as 3β-acetoxy-4α,5-epoxy-5α-cholestan. In another attempt to identify 4α,5-epoxy-5α-cholestan-3β-ol, the compounds at C5 were reduced with lithium aluminium hydride and analyzed with TLC-RD in system 1. Radioactive spots were detected at the R_f-values for 5α-cholestane-3β,5-diol and 5β-cholestane-3β,5-diol (see Table I) in yields of 10% and 42%, respectively. Extraction of these radioactive spots followed by GLC-RD analysis on an SP-2100 column yielded radioactive peaks at the t_R-values for the TMS ethers of the respective diol (see Table I). Lithium aluminium hydride reduction of the authentic 4,5-epoxides afforded only 5α-cholestane-3β,5-diol (80% yield) from the α-epoxide and only 5β-cholestane-3β,5-diol (74% yield) from the β-epoxide (19). No further attempts were made to identify 4α,5-epoxy-5α-cholestan-3β-ol. The yields of compounds identified from LC fraction II are listed in Table II.

The major compounds identified from LC fraction III after LC were 4-cholesten-3-one and 4-cholesten-3β-ol. They were identified according to the standard procedure. The yields were 44 and 32%, respectively.

Products of 4-Cholesten-3α-ol

The yields of radioactivity from LC fractions I and II were 2 and 10%, respectively. The metabolite pattern from LC fraction II is shown in Figure 1. Compounds D2 and D4 were found to be identical to 4-cholestene-3α,6β-diol and 6β-hydroxy-4-cholesten-3-one. GLC-RD analysis of the TMS ether of compound D1 afforded a single radioactive peak at t_R = 2.18. The t_R-value for the TMS ether of 4-cholestene-3α,6β-diol was 2.04 but GLC-MS analysis of compound D1 yielded a mass spectrum that was only slightly different from that of the TMS ether of 4-cholestene-3α,6β-diol. Compound D1 was therefore identified as 4-cholestene-3α,6α-diol. Compound D3 was identified as 6α-hydroxy-4-cholesten-3-one as just described for compound C3. Compounds D5 and D6 were identified as 4α,5-epoxy-5α-cholestan-3α-ol and 4β,5-epoxy-5β-cholestan-3α-ol, respectively, according to the standard procedure. The yields of compounds identified from LC fraction II are listed in Table II.

The major compounds identified from LC fraction III were 4-cholesten-3-one and 4-cholesten-3α-ol. They were identified according to the standard procedure. The yields were 15 and 63%, respectively.

Products of 4-Cholesten-3-one and 4-Stigmasten-3-one

The yields of radioactivity from LC fractions I and II were 2 and 5%, respectively, for both substrates. Compound E2 (Fig. 1) from LC fraction II was identified according to the standard procedure as 6β-hydroxy-4-cholesten-3-one. Compound E1 was identified as 6α-hydroxy-4-cholesten-3-one as described for compound C3 obtained from incubations with 4-cholesten-3β-ol.

GLC-RD analyses of each of the TMS ethers of compounds F1 and F2 on an SP-2100 column afforded a single radioactive peak at t_R = 5.69 (F1) and t_R = 4.28 (F2), i.e., 1.57 times the t_R-value for 6α-hydroxy-4-cholesten-3-one and 6β-hydroxy-4-cholesten-3-one, respectively. In previous studies, C_{29}-steroids oxygenated in the A and B rings constantly had t_R-values on an SP-2100 column that were 1.5-1.6 times those of the analogous C_{27}-steroids (6,20). Compounds F1 and F2 were therefore tentatively identified as 24α-ethyl-6α-hydroxy-4-cholesten-3-one and 24α-ethyl-6β-hydroxy-4-cholesten-3-one, respectively. GLC-MS analysis confirmed the preliminary identifications yielding mass spectra that only differed from those of the C_{27}-analogs in that the side chain containing fragments were found 28 mass units above those of the C_{27}-compounds. The yields of the compounds identified from LC fraction II are listed in Table II.

LC fraction III contained unchanged 4-cholesten-3-one and 4-stigmasten-3-one, respectively (identified according to the standard procedure) and the yields were 83% for both compounds. Traces of less polar (TLC-RD) compounds than 6β-hydroxy-4-cholesten-3-one were seen in both LC fractions II and III. Identification of 4α,5-epoxy-5α-cholestan-3-one and 4β,5-epoxy-5β-cholestan-3-one was unsuccessful. If these compounds were formed, the yields of each one must have been less than 0.02%.

Metabolites from Incubations with Rat Liver Subcellular Fractions

The similarities in metabolism between cholesterol and epicholesterol that were found

after experiments with soybean lipoxygenase were also seen following incubations with iron-supplemented rat liver microsomes (Table II). The major metabolites formed were also identified after incubations of cholesterol and epicholesterol with rat liver 18,000 × g supernatant fractions. However, with the exception of 5-cholestene-3β-7α-diol (from cholesterol), much lower yields of metabolites were recorded with the cholesterol and epicholesterol incubated with rat liver 18,000 × g supernatant.

As can be deduced from Table II, the 18,000 × g supernate caused epoxidation of all unsaturated sterol substrates. As with the soybean system, the 2 Δ^5-steroids were epoxidized to the same extent. Unlike the findings with the soybean system, the epoxidation of 4-cholesten-3α-ol occurred in higher yields than with the 2 Δ^5-steroids, and the formation of 4,5-epoxides of 4-cholesten-3β-ol could barely be demonstrated.

DISCUSSION

In this study with soybean lipoxygenase, the formation of C-7 oxygenated metabolites from epicholesterol was qualitatively and quantitatively similar to that found for cholesterol. The influence of the hydroxyl group at C-3 on the yields of 7-oxygenated metabolites thus seems to be negligible. As with cholesterol, an intermediary formation of 7-hydroperoxides seems to be involved (3,4). These reactions with soybean lipoxygenase have been suggested to involve radical processes and not singlet oxygen (3,4). A participation of superoxide radical anion in cholesterol oxidation has previously been excluded by Smith et al. (21).

The formation of 6β-hydroxy-4-cholesten-3-one in similar yields (0.3%) from both cholesterol and epicholesterol cannot likely result from an intermediary formation of 4-cholesten-3-one which was isolated in less than 1% yields. When the 4-cholesten-3-one was incubated with soybean lipoxygenase, 6β-hydroxy-4-cholesten-3-one was isolated in only 0.9% yields. However, Kulig and Smith (13) have reported on the formation of 3β-hydroxy-4-cholesten-6β-hydroperoxide from cholesterol. It is possible that this compound (or the 3α-isomer) was the precursor of 6β-hydroxy-4-cholesten-3-one obtained from soybean lipoxygenase oxidation of cholesterol and epicholesterol.

The identification of 5,6-epoxycholestan-3α-ol was based primarily upon the mass spectrum of the TMS ether of this compound, which was identical to that of the TMS ether of 5,6β-epoxy-5β-cholestan-3β-ol (and 5,6α-epoxy-5α-cholestan-3β-ol). Since there is no significant difference between the mass spectra of the 2 5,6-epoxides of cholesterol, it cannot be evaluated from the mass spectrum which isomer was formed from epicholesterol. It may be that the identified epoxide is composed of a mixture of both isomers.

The 3-hydroxy-Δ^4-steroids yielded 6-hydroxy- and 5,6-epoxy-derivatives as the major dioxygenated metabolites. 4-Cholesten-3α-ol and 4-cholesten-3β-ol afforded also 4-cholesten-3-one and its metabolites 6α-hydroxy-4-cholesten-3-one and 6β-hydroxy-4-cholesten-3-one. Whether 6α-hydroxy-4-cholesten-3-one and 6β-hydroxy-4-cholesten-3-one were formed via 4-cholesten-3-one or via 4-cholesten-3,6-diols cannot be stated. The ratio between 6β- and 6α-hydroxylated metabolites ranged between 2 and 3.

Although the 4β,5β-epoxide was the dominant isomer formed from 4-cholesten-3β-ol, 4-cholesten-3α-ol afforded the 2 4,5-epoxides in similar yields. These epoxy-derivatives were not found during recent studies by Teng and Smith (5) on the oxidation of 4-cholesten-3β-ol and 4-cholesten-3-one by soybean lipoxygenase, nor was the formation of 4-cholestene-3β,6α-diol or 4-cholestene-3β,6β-diol reported. The possibility remains that the 6-hydroxylated and epoxidized products of 4-cholesten-3β-ol found in this study originate from 6-hydroperoxides of 4-cholesten-3β-ol (22). Slight differences in the methodology used (pH of the incubation medium and work-up procedures) as well as heterogeneity of commercial soybean lipoxygenase (23,24) might explain the difference in results between this study and that of Teng and Smith. These investigators have suggested that oxidation of 4-cholesten-3β-ol by soybean lipoxygenase is caused by nonspecific free radical processes (5). The lack of formation of 4α,5-epoxy-5α-cholestan-3-one was considered as partial evidence to exclude participation of singlet molecular oxygen. In this study, it was possible to confirm that neither 4-cholesten-3α-ol, 4-cholesten-3β-ol, nor 4-cholesten-3-one afforded 4α,5-epoxy-5α-cholestan-3-one or 4β,5-epoxy-5β-cholestan-3-one.

The steroid metabolism patterns obtained with soybean lipoxygenase and iron-supplemented microsomes were found to be very similar. The preparations of iron-supplemented microsomes that were used have been shown to stimulate nonspecific lipid peroxidation (17,25). When conditions which have been found to minimize this activity in the microsomes (17) were used, some reactions still took place to a significant degree. The 6β-hydroxylation of 4-cholesten-3-one and 4-stigmasten-3-one is one example, and it is tempting to

suggest that the microsomes contain a 6β-hydroxylase. The simultaneous absence of 6α-hydroxylated metabolites, which were found to be obligatory companions to the 6β-hydroxy isomers in iron-supplemented microsomes and soybean lipoxygenase oxidations, lends further support to the concept that microsomal fractions from rat liver contain a C_{27}-steroid 6β-hydroxylase.

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The Use of 13-Methyltetradecanoic Acid As an Indicator of Adipose Tissue Turnover

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ABSTRACT

We show in this paper that 13-methyltetradecanoic acid (13-MTD) can be used as a structurally labeled marker for investigating the mobility of fatty acyl chains in adipose tissue in the rat. The presence of an ω-1 methyl group allows easy quantitation by gas liquid chromatography (GLC) and permits an assessment to be made of any oxidation and chain elongation reactions with reincorporation of the label into the adipose tissue, since the iso-acyl chain is well resolved from odd or even-numbered homologous fatty acids with straight chains. The kinetics of uptake and loss of the structural label were different for adipose tissue taken from the various sites which were sampled, namely post abdominal, mesenteric, perirenal, pericardiac and subcutaneous adipose tissue as well as the epidydimal fat pads. We also report preliminary results in man which confirm that the method is applicable to human clinical studies and that 13-MTD kinetics differ for adipose tissue taken from the 3 different subcutaneous sites–waist, arm and thigh.

NOMENCLATURE

13-MTD, 13-methyltetradecanoic acid; 13-MTDG, 13-methyltetradecanoin tri-[13-methyltetradecanoyl]-glycerol).

INTRODUCTION

The study of plasma free fatty acid (FFA) turnover (1) or aspects of it such as clearance, oxidation or uptake by different tissues, has been the object of numerous investigations in both man and animals (2-5). Few studies, however, have dealt with the direct incorporation of dietary fatty acids into, and their subsequent release from adipose tissue. A major obstacle in this respect is that the measurement of adipose tissue fatty acid turnover in vivo using radioactively labeled material is ethically limited to animal studies. The use of large amounts of specifically labeled ^{13}C-fatty acids is not feasible on economic grounds. However, the use of a structurally labeled fatty acid occurring naturally in trace amounts and not synthesized de novo by the body would provide a method which was more applicable to clinical studies in man.

Among the few investigators who have attempted to use such nonradioactive labels, Garton et al. (6) fed lambs with either acetate, butyrate or propionate mixed with a conventional diet and demonstrated a significant incorporation of odd-carbon normal saturated fatty acids as well as monomethyl-branched fatty acids into the adipose tissue of those animals reared on the propionate-containing diet. Pi-Sunyer (7) fed rats with triundecanoin, an 11:0 triglyceride, for 6 weeks and showed a marked incorporation of undecanoate and longer odd-carbon acids including tridecanoic and pentadecanoic acids into their adipose tissue fat stores. Campbell and Hashim (8,9) had also observed chain elongation upon feeding triundecanoin to both dogs and rats and advocated the use of an odd-carbon fatty acid-enriched diet for the study of depot fatty acid turnover.

The purpose of this study was to develop a method of labeling the adipose tissue suitable for clinical studies in man. Medium chain length fatty acids up to C10-12 are absorbed by the intestine and to a large extent transported by the portal circulation (10-12) to be oxidized in the liver (13,14), with only a small proportion being transported as chylomicra in the lymph and therefore available for incorporation into the adipose tissue glyceride pool. This led us to use a longer chain length fatty acid known to be readily transported in the lymph as chylomicra (12,15-17), in order to overcome this particular problem. We chose to use 13-methyltetradecanoic (isopentadecanoic) acid, double labeling providing us with significant improvements over the use of an odd-carbon chain alone as a structural label for identifying its incorporation into adipose tissue. The presence of the isopropyl group makes it possible to identify readily both chain elongation and α- or β-oxidation products by means of the group's characteristic influence on gas chromatographic behavior, as well as distinguishing this group from a straight chain ω-terminus using mass

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spectrometry (MS) and nuclear magnetic resonance (NMR) spectoscopy. It also enabled us to assess any recycling of the labeled fatty acid that might have occurred.

13-Methyltetradecanoic acid (13-MTD) is not synthesized de novo in the body but occurs naturally in both bovine (18) and human milk (19) as well as in various ox and sheep depot fats together with closely related branched-chain iso and anteiso odd-numbered acids (20-25). Moreover, it is possible to prepare 13-methyltetradecanoic acid by an unambiguous synthetic route in the large quantities necessary for dietary experiments.

We decided to explore the use of 13-MTD as an indicator of adipose tissue fatty acid turnover in rats solely on grounds of convenience before applying the method, if shown to work successfully in a laboratory animal, to studies on human adipose tissue metabolism. In this paper, we report the synthesis and characterization of the triglyceride-containing 13-MTD (13-MTDG), and the results of dietary experiments using rats fed with a 13-MTDG supplement. Some preliminary results obtained with human volunteers are also described demonstrating that the method is applicable to human clinical studies.

MATERIALS AND METHODS

Synthesis of 13-Methyltetradecanoic Acid (13-MTD)

13-Methyltetradecanoic acid was synthesized electrolytically from isovaleric acid and methyl hydrogen dodecanedioate in methanolic solution (26-28). Dimethyl dodecanedioate was prepared from commercially available dodecanedioic acid (Aldrich Chemical Company, Milwaukee, WI), by esterification with 75 parts v/w methanol containing 5% w/v concentrated sulfuric acid. The dimethyl ester, after purification by vacuum distillation, was converted to the half ester using the theoretical quantity of anhydrous barium hydroxide in absolute methanol (29). The methyl hydrogen dodecan-1,12-dioate was purified by distillation.

The electrolytic coupling reaction was carried out with monoester (typically 3-5 mol) and a 2–fold molar excess of isovaleric acid dissolved in methanol (12 ℓ) containing sodium methoxide (5 g), using 15 cm × 10 cm platinum electrodes at a current density of 0.053-0.066 A dc cm^{-2}. The reaction mixture was stirred magnetically and maintained at 50 C by water cooling. Electrode polarity was reversed every 25 min to prevent the build-up of deposits on the electrode surfaces. The point at which the gas liquid chromatography (GLC) peak for methyl 13-MTD no longer increased provided a more reliable endpoint than either the solution going alkaline or the passage of the theoretical number of coulombs. Although we used carbon plates originally for economic reasons, we found that platinum electrodes were essential in order to keep side reactions to a minimum. With carbon electrodes, an equivalent amount of a second compound apart from methyl 13-MTD was produced, and its GLC behavior indicated it was probably methyl 12-methyltetradecanoate, suggesting free radical migration (26,28).

After electrolysis the reaction mixture was cooled to room temperature and the dimethyl docosanedioate which precipitated was removed by filtration. The filtrate was acidified with acetic acid and the methanol removed by rotary evaporation under reduced pressure. The crude methyl 13-MTD was purified by fractional vacuum distillation. Analytical data were as follows: Dimethyl dodecanedioate–bp 176-182 C/3-5 mm (lit. 150 C/2 mm); 100 MHz NMR chemical shifts: $C\bar{H}_3O$, 3.68δ (singlet, 6H); $C\bar{H}_2CO$, 2.32δ (triplet, 4H, J=6-7 Hz); $C\bar{H}_2CH_2CO$, 1.64δ (triplet, 4H, J=6-7 Hz); $(-C\bar{H}_2-)$, 1.33δ (singlet, 12H). Methyl hydrogen dodecanedioate–bp 198-204 C/5-7 mm. 100 MHz NMR chemical shifts: $C\bar{H}_3O$, 3.68δ (singlet, 3H); $C\bar{H}_2CO$ (ester), 2.32δ (triplet, 2H, J=7 Hz); $C\bar{H}_2CO$ (acid), 2.36δ (triplet, 2H, J=7 Hz); $C\bar{H}_2CH_2CO$, 1.64δ (triplet, 4H, J=6.5 Hz); $(-C\bar{H}_2-)$, 1.32δ (singlet, 12H); $COO\bar{H}$, 11.34δ (singlet, 1H). Methyl 13-methyltetradecanoate (13-MTD)–bp 138-144 C/2-3 mm (lit. 100-106 C/0.3 mm; estim. 135 C/2 mm); C=75.31%; H=12.69%; 0=12.25% (74.94, 12.58, 12.48 calculated); 100 MHz NMR chemical shifts: $C\bar{H}_3O$, 3.66δ (singlet, 3H); $C\bar{H}_2CO$, 2.31δ (triplet, 2H, J=7.5 Hz); $(-C\bar{H}_2-)$, 1.29δ (singlet, 20H); $C\bar{H}_2CH_2CO + C\bar{H}$, 1.62δ (triplet, 3H, J=6 Hz); $(C\bar{H}_3)_2CH$, 0.87δ (doublet, 6H, J=6 Hz). The 70 eV mass spectrum gave M^+, m/e 256; $(M-29)^+$, m/e 227; $(M-31)^+$, m/e 225; $(M-43)^+$, m/e 213; base peak m/e 74. Accurate mass measurement for M^+ ($C_{16}H_{32}O_2$) gave m/e 256.23999 (calculated mass 256.24020). Infrared (IR) spectroscopy showed C=O, 1745 cm^{-1} (stretch); and $(CH_3)_2CH$, 1365 cm^{-1} and 1383 cm^{-1} (deform.). GLC analysis gave a single peak with equivalent chain length as follows: on PEGA (197 C), 15.40; on SE30 (200 C), 15.30.

Synthesis of Tri-(13-methyltetradecanoyl)-glycerol (13-MTDG)

Methyl 13-methyltetradecanoate was hydrolyzed by refluxing with excess 10% w/v sodium

hydroxide in ethanol/water (50:50, v/v). After cooling and acidification, the free acid was extracted with diethyl ether and purified by vacuum distillation. 13-Methyltetradecanoyl chloride was prepared by refluxing anhydrous 13-MTD with excess oxalyl chloride for 2 hr, removing the oxalyl chloride by distillation at atmospheric pressure and purifying the acid chloride by vacuum distillation. The acid chloride was added with efficient stirring to anhydrous ethanol-free chloroform containing 1 mol equivalent of dry pyridine and the theoretical quantity of anhydrous glycerol. Stirring was continued for 10 hr and the mixture allowed to stand overnight at room temperature. The reaction mixture was then refluxed for 2 hr, allowed to cool, and the chloroform solution successively washed with 1 N hydrochloric acid, methanol/water (50:50, v/v), 1 M potassium bicarbonate and water, before being dried over anhydrous sodium sulfate. The solution of crude triglyceride in chloroform was treated with activated charcoal and the chloroform then removed under reduced pressure by rotary evaporation.

The crude 13-MTDG was purified by passing a solution of it in chloroform over silicic acid (Mallinckrodt CC4), followed by rechromatography of the triglyceride fraction over silicic acid using diethyl ether/petroleum ether (40-60 C fraction) 25:75, v/v. The eluate was decolorized with activated charcoal and the solvent removed leaving a light-yellow oil. The partially purified triglyceride was recrystallized from ethanol/diethyl ether (6:1, v/v). The product, which solidified at room temperature, gave a major spot (> 95%) on TLC: silica gel, petroleum ether 40-60 C fraction/diethyl ether/acetic acid (80:20:1 v/v). Minor impurities (< 5%) corresponding to 1,2- and 1,3-diglycerides, monoglycerides and fatty acids were detected. The fatty acid content of the glyceride determined by GLC was >99% 13-methyltetradecanoic acid. Analytical data were as follows: 13-methyltetradecanoic acid (13-MTD)–bp 164-167 C/2 mm (lit. n-15:0 158 C/1 mm). 13-Methyltetradecanoyl chloride–bp 162-178 C/8-9 mm (lit. n-15:0 157 C/5 mm); n_D^{20} =1.4502; IR spectroscopy showed C=O, 1802 cm^{-1} (stretch). Tri-(13-methyltetradecanoyl)-glycerol (13-MTDG)–C=75.58%; H=11.87%; O=12.40% (75.34,12.12.12.54 calculated). IR spectroscopy showed $(CH_3)_2CH$, 1365 cm^{-1} and1382 cm^{-1} (deform.); C=O, 1740 cm^{-1} (stretch). 100 MHz NMR chemical shifts: $(C\bar{H}_3)_2CH$, 0.89δ (doublet, 18H, J=6 Hz); $(-C\bar{H}_2-)$, 1.31δ (singlet + shoulder, 63H); $C\bar{H}_2CO$, 2.34δ (triplet, 6H, J=7 Hz); $C\bar{H}_2O$, 4.25δ ($[AB]_2X$ octet, 4H, J_{ab}=12 Hz, J_{ax}=4.2 Hz, J_{bx}=5.9 Hz); $C\bar{H}O$, 5.28δ (AX_4 perturbed quintet, 1H, J=5 Hz).

The 70 eV mass spectrum showed M^+, m/e 765;$(M\text{-}RCO_2)^+$, m/e 523; $(M\text{-}RCO_2CH_2)^+$, m/e 509. Accurate mass measurement gave M^+ ($C_{48}H_{92}O_6$) m/e 764.69174 (calculated mass, 764.68934), and $(M\text{-}RCO_2CH_2)^+$ ($C_{32}H_{61}O_4$) m/e 509.45701 (calculated mass, 509.45695). Methyl ester content estimated by NMR was 0.3% by weight.

Overall yields in the synthesis of 13-MTDG were 6.6% and 12.6% for 2 separate preparations (dimethyl dodecanedioate = 100%). Stage yields for the conversion of 13-MTD to 13-MTDG were 33% and 43%, respectively. At all stages yield was sacrificed for the highest purity obtainable.

Dietary Composition

Postweanling rats (male, CFY strain) were fed a diet contaning 20% protein, 10% fat and 64% carbohydrate by weight and adequate minerals and vitamins to sustain optimal growth (D.W.T. Crompton, personal communication). The diet had a calculated calorific content of 3.86 ME cal g^{-1} and was fed at the rate of 15 g/day for 2 weeks postweaning and 20 g/day thereafter. The experimental group of animals received the 13-MTDG supplement for 3 weeks.

Preparation of the Corn Oil plus 13-MTDG Mixture

One part by vol of 13-MTDG was mixed with 9 vol of commercially available corn oil (Boots Pure Drug Co., Nottingham) and the fatty acyl chains randomized by heating the mixture to 80-100 C for 2 hr in the presence of 0.1% sodium methoxide under an atmosphere of nitrogen (30,31). Randomization was checked by argentation chromatography. The fatty acid composition of the corn oil-13-MTDG mixture is given in Table I.

Sampling of Adipose Tissue

Groups of animals were killed under ether

TABLE I

GLC Analysis of Dietary Fat Supplement Containing Corn Oil plus 13-MTDG

Fatty acid	Composition (wt %)
iso-15:0	6.2
n-16:0	10.1
n-18:0	1.6
*c*9-18:1	24.6
*c*9,12-18:2	56.5
*c*9,12,15-18:3	0.7

anesthesia at the following times after starting on the diet: zero weeks (controls, 6 animals); 1,2,3,4,6 and 10 weeks (4 animals each); Weights were recorded every other day. A similar group of rats maintained on a diet containing corn oil alone were sampled at similar intervals to provide baseline data.

Samples of adipose tissue (100-500 mg) were removed by dissection from the following anatomical locations: epididymal fat pad, subcutaneous, mesenteric, postabdominal, perirenal and pericardiac adipose tissue. Blood was taken by aortic or cardiac puncture into heparinized tubes and the plasma separated by centrifugation. Tissue was also removed for standard histological assessment of possible toxic side effects. Pieces of adipose tissue or an aliquot of plasma were immediately placed into 10 ml of chloroform/methanol (2:1,v/v) (32) and dispersed with an all-glass or PTFE-glass homogenizer. Two ml 145 mM NaCl solution were added, the mixture shaken well and the phases allowed to separate. The protein precipitate was removed by filtration through clean glass wool and the aqueous phase removed. The chloroform phase, after washing with methanol/145 mM NaCl (1:1, v/v), was dried under a stream of nitrogen and the residue dissolved in a small volume of chloroform/methanol (2:1, v/v) for analysis.

Preparation of Fatty Acid Methyl Esters

Fatty acid methyl esters were prepared from adipose tissue lipids by alkoxide-catalyzed methanolysis (33) and dissolved in *n*-hexane for GLC analysis.

Human Studies

Two human volunteers (one normal male, P.P., and one obese female, T.L.) were fed an appropriate corn oil-plus-13-MTDG supplement as part of a rigorously controlled diet. Samples of adipose tissue from the arm, thigh and waist were obtained by percutaneous needle biopsy. All experiments on human volunteers were conducted with prior consent of the Ethical Committee (EC 13), Clinical Research Centre and Northwich Park Hospital, Harrow, Middlesex HA1 3UJ, U.K,

Gas Chromatography and Mass Spectrometry

Fatty acid methyl esters were analyzed on columns of polyethylene glycol adipate (PEGA; 330 cm x 6mm) using argon as carrier gas at 30 ml min^{-1} and flame ionization detection in a Pye Series 104 gas chromatograph. The composition of each sample was determined either using peak height and retention time (34) or by electronic integration. Gas chromatography-mass spectrometry (GC-MS) was conducted using a Pye 104 coupled to an Associated Electrical Industries MS902 double-focusing mass spectrometer with an ionization energy of 70 eV as previously described (35).

Reagents

All reagents were of AR grade or the equivalent; bulk chemicals were obtained in the purest commercial grade available.

RESULTS AND DISCUSSION

No significant differences were observed in weight gain for groups of rats fed corn oil alone or corn oil plus 13-MTDG, suggesting that its inclusion had not altered the overall use of available calories. We found no histological evidence of organ toxicity resulting from the administration of 13-MTDG at any stage of the experiment. Histological assessment of a range of tissues including liver, kidney, spleen, brain and cardiovascular tissue was carried out whenever adipose tissue was sampled for analysis.

GC analysis of fatty acid methyl esters prepared from subcutaneous adipose tissue is illustrated in Figure 1. This demonstrates the increase in the peak for 13-MTD methyl ester after 3 weeks on the diet, followed by the decrease 3 weeks after removing the 13- MTDG supplement. Also shown in Figure 1 is the excellent resolution of this peak from surrounding fatty acid methyl esters. Since the terminal isopropyl group enables one to distinguish 13-MTD and related fatty acid methyl esters from straight-chain saturated and unsaturated homologs, we were in a position to determine whether α- and β-oxidation, chain elongation or desaturation with reincorporation of acyl chains into the adipose tissue occurred. We found no evidence by GLC that any of these metabolic interconversions took place with reincorporation into the adipose tissue glyceride pool to any detectable extent. By contrast, both chain elongation and desaturation have been observed during the feeding of triundecanoin to dogs (8).

We felt it was essential to demonstrate that the GLC peak which increased as a result of feeding corn oil plus 13-MTDG (Figure 1) was indeed caused by the methyl ester of 13-MTD. This was shown to be the case for samples taken at random from among those we analyzed. Using GC-MS we demonstated that the fatty acid incorporated into adipose tissue was 13-methyltetradecanoic acid (13-MTD) on

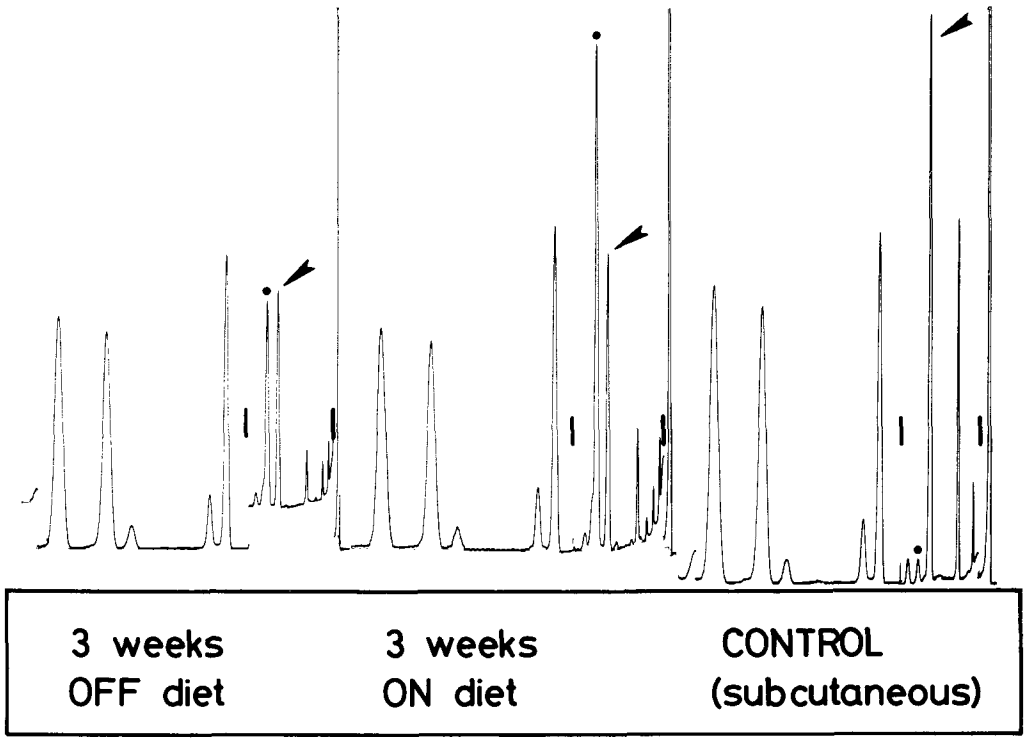

FIG. 1. GLC of fatty acid methyl esters prepared from rat subcutaneous adipose tissue showing the incorporation and loss of the 13-MTD label administered in the diet. Methyl 13-MTD (•) and methyl myristate (⊀) are specifically indicated. The separation was carried out on columns of polyethylene glycol adipate at 197 C as detailed in Materials and Methods.

the basis of the mass spectrum of its methyl ester being identical with the synthesized material. Also, the incorporated material exhibited GLC behavior on PEGA and SE30 columns characteristic of an ω-1 methyl-branched fatty acid methyl ester. The majority of the lipid extracted from adipose tissue is in the form of triglyceride (36-40). Thin layer chromatography (TLC) analysis of a selection of our samples showed that >95% of the total extractable lipids were triglycerides.

The incorporation of 13-MTD into the lipids extracted from subcutaneous, postabdominal, mesenteric, epididymal, perirenal and pericardiac adipose tissue is shown in Figure 2 (a-f). No detectable increases in 13-MTD levels in plasma were found, suggesting extremely rapid clearance. These results are given as the absolute percentage by weight of 13-MTD in the total adipose tissue fatty acids, with the size of the symbols representing the range of values obtained. Although the number of points in Figure 2 are limited, it is possible to distinguish the kinetics of uptake and loss of 13-MTD label for the 6 tissues sampled. The t½ for uptake was very similar for all the tissues, lying between 4½-6½ days. Loss of 13-MTD label is, however, characterized by somewhat greater differences between the tissues with t½ ranging from 9-10 days (pericardiac and perirenal) to 18 days (epididymal). Postabdominal and mesenteric adipose tissue are very similar (11-12 days) whereas subcutaneous adipose tissue resembles epididymal fat in its t½ for loss of label (15-16 days). There is some suggestion from the semilogarithmic plots that there may be a second exponential phase with t½ greater than 30 days. Although the limited data reported here do not permit a definite conclusion, it is worth noting that a 2-compartment system for fatty acid uptake has been suggested by previous work (41).

Comparable values of t½ have been observed for the uptake and loss of undecanoate by perirenal adipose tissue in rats fed triundecanoin (uptake t½ = 8.7 days; loss t½ = 12.1 days) by Campbell and Hashim (9). These half-life times are generally rather shorter than other reported values for fatty acids in adipose tissue using radioactive or structural markers

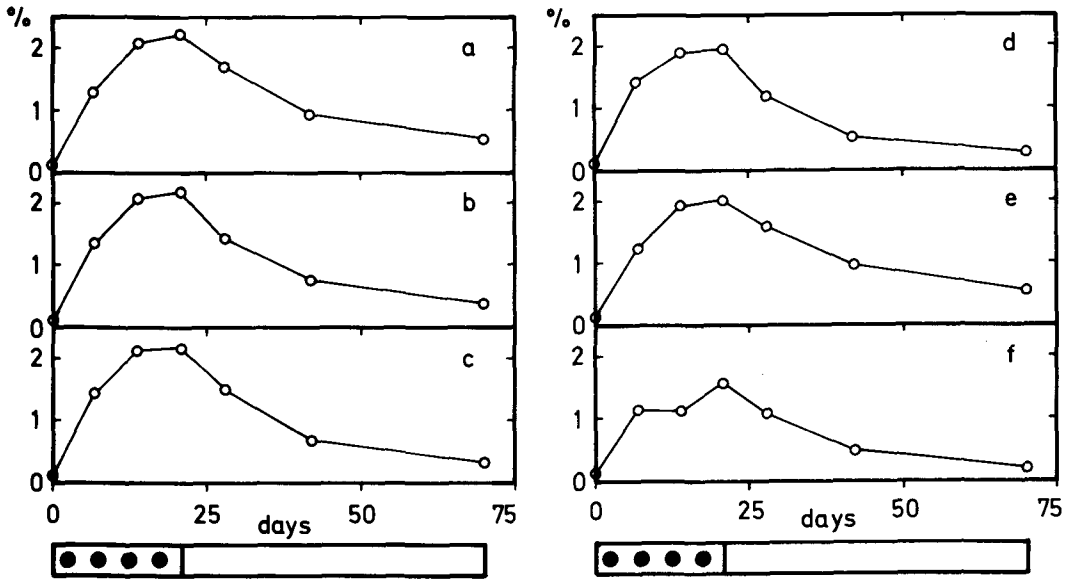

FIG. 2. Incorporation of 13-MTD label into adipose tissue from various sites in the rat, shown as the absolute percent by weight of the total fatty acids. The period of feeding with 13-MTDG is shown as •: (a) subcutaneous; (b) postabdominal; (c) mesenteric; (d) perirenal; (e) epididymal; (f) pericardiac.

(41). This discrepancy may arise because of extensive recycling and metabolism of the labels used in these studies.

We can offer no satisfactory explanation for the somewhat peculiar kinetics of uptake shown by pericardiac tissue (Fig. 2f). This peculiarity was not reflected in the other tissues examined at the same time. The very small mass of pericardiac adipose tissue may render it more liable to short-term changes in composition than the larger, more "buffered" sites. Although differences in the kinetics of 13-MTD loss were observed between the various tissues, the overall fatty acid compositions were remarkably similar at different stages in the experiment (Table II) with the possible exception of pericardiac fat. Samples taken from right and left sides showed no significant

TABLE II

Fatty Acid Composition by Weight of Adipose Tissue Taken from Various Anatomical Sites in the Rat[a]

	Fatty Acid (wt %)						
	14.0	16:0	16:1	18:0	18:1	18:2	18:3
Subcutaneous	(A) 1.3	19.3	4.1	2.8	30.4	41.1	0.6
	(B) 0.6	16.9	2.4	3.2	31.5	44.3	0.5
Postabdominal	(A) 1.2	22.4	5.1	2.7	29.7	38.3	0.6
	(B) 0.6	18.8	3.1	3.2	31.4	41.9	0.5
Mesenteric	(A) 1.4	22.1	5.1	2.9	29.2	38.9	0.5
	(B) 0.6	18.2	2.8	3.3	32.4	41.8	0.5
Perirenal	(A) 1.2	23.4	5.7	3.0	30.6	35.3	0.7
	(B) 0.8	20.8	3.8	3.8	32.6	37.4	0.5
Epididymal	(A) 1.3	21.0	5.9	2.5	29.4	39.2	0.5
	(B) 0.7	19.0	4.5	2.6	30.5	41.4	0.7
Pericardiac	(A) 1.7	22.8	4.0	6.4	30.3	29.5	0.8
	(B) 0.5	19.9	3.7	3.9	34.7	36.1	0.5

[a]Only major components are shown (≥ 0.5%): (A) immediately at the start of the experimental diet; (B) 10 weeks later. Reproducibility between animals was ca. 2-3% of the value quoted.

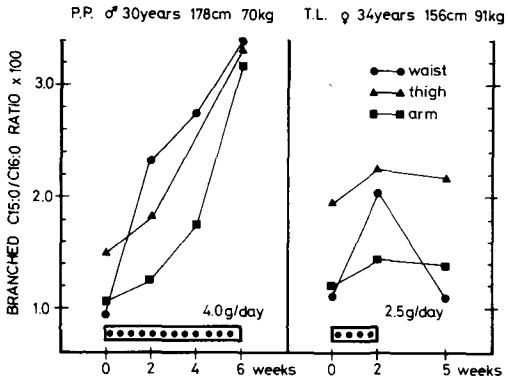

FIG. 3. Incorporation of 13-MTD label into human adipose tissue from arm, waist and thigh. Palmitate was used as an internal standard. The period of feeding with 13-MTDG is shown as •, and the amount per day represents 13-MTDG added to the diet.

differences.

Data for 13-MTD uptake by human adipose tissue are shown in Figure 3, in which the level of tissue palmitic acid is used as an internal standard. These results strongly suggest that subcutaneous adipose tissue in man around the waist is more mobile as far as labeling with 13-MTD is concerned than that of either the upper arm or thigh. A simple estimate of the relative rate of labeling may be obtained by comparing the incorporation of 13-MTD at 2 weeks with that after 6 weeks, giving ratios of 0.56, waist; 0.12, thigh; 0.09, arm (derived from the data for P.P.). Both sets of data indicate the relative lability of subcutaneous adipose tissue around the waist. Further human clinical studies are already in progress using 13-MTD labeling as a means of investigating subcutaneous adipose tissue turnover in man.

In summary, we have shown that 13-MTD may be used to label adipose tissue glycerides in vivo. Uptake and loss of the label is easily followed by GC. There is no evidence for the metabolism and recycling of 13-MTD in adipose tissue and we believe, therefore, that 13-MTD is preferable to other structurally or radioactively labeled markers which may be metabolically altered and reincorporated, as a means of estimating adipose tissue turnover. Reincorporation of the marker would lead to artificially long biological half-lifes.

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Cuticular Lipid Constituents of Cabbage Seedpod Weevils and Host Plant Oviposition Sites As Potential Pheromones

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ABSTRACT

The cuticular lipids of cabbage seedpod weevils (*Ceutorrhynchus assimilis* Payk.) and those at their oviposition site, i.e., the seed pods of the host plant (*Brassica napus* L.), were analyzed by chromatographic techniques in conjunction with mass spectrometry (MS). Long chain hydrocarbons were most abundant in both lipids; however, the seed pods of *B. napus* contained *n*-alkanes only, whereas *C. assimilis* showed a predominance of dimethylalkanes over internally branched methylalkanes as well as iso- and *n*-alkanes. The amounts of ketones, secondary alcohols and aldehydes, the usual plant components that are unique in insects, occurred in cuticular lipids of both organisms in approximately the same ratio. The composition of *n*-alkanes, *n*-ketones, secondary *n*-alcohols, iso- and anteiso-aldehydes and esterified primary anteiso-alcohols of wax esters was similar between *C. assimilis* and *B. napus*. In both sources, qualitative but not quantitative similarities were observed in the composition of *n*-aldehydes and esterified primary *n*- and iso-alcohols, respectively. The esterified fatty acids of wax esters from *B. napus* were composed of roughly equal proportions of saturated branched and unbranched components. The esterified fatty acids of wax esters and steryl esters from *C. assimilis* consisted of major proportions of saturated as well as unsaturated *n*-compounds, whereas iso- and anteiso structures were present in minor proportions only. Free fatty acids and traces of ethyl esters of fatty acids found in *C. assimilis* were mainly composed of unsaturated *n*-compounds.

INTRODUCTION

Interactions of phytophagous insects with their host plants in terms of chemical messengers, such as insect pheromones or related behavior-modifying chemicals, are being slowly unravelled (1-3). It is becoming increasingly apparent that surface lipids play an important role in the chemical communication between members of the same species or members of different species (4). Surface waxes from various insects (5) as well as from higher plants (6) have been investigated. Close-range sex pheromones have been found in the cuticular lipids of flies (7-21), midges (22), cockroaches (23,24) and beetles (25-27). Insect attractants from host plants may serve insects as key chemical cues for locating food resources and oviposition sites (28).

The cabbage seedpod weevil, *Ceutorrhynchus assimilis* Payk., causes considerable damage to the cultivation of rape, *Brassica napus* L., by puncturing the seed pods for oviposition, and from frass by larvae and adults. The beetles, after hibernation, are guided to rape as their principal host plant by a combination of visual and olfactory stimuli (29). In this study, the compositions of surface lipids of *C. assimilis* and seed pods of *B. napus* (cv. Lesira) were investigated for the presence of insect-host plant interrelated constituents that might play a role as key chemical cues in reproduction of the beetle.

MATERIALS AND METHODS

All solvents were analytical grade and were redistilled before use. Adsorbents for thin layer chromatography (TLC) and reagents were purchased from E. Merck AG, Darmstadt, Germany. Lipids used as reference compounds for chromatography were obtained from Nu-Chek-Prep, Inc., Elysian, MN, from ICN Pharmaceuticals, Inc., Plainview, NY, and from Applied Science Laboratories, Inc., State College, PA.

Isolation of Cuticular Lipids

Sexually mature beetles *C. assimilis* Payk. (ca. 3 mm long) as well as immature seed pods (2-6 cm long) of host plants *B. napus*, cv. Lesira, were collected during April to June from field-grown plants. The cuticular lipids of insects and seed pods were washed off separately by a 1-min immersion into excess hexane and then isolated by filtration over glass wool.

Separation and Quantification of Lipid Classes

Cuticular lipids were fractionated into lipid classes by chromatography on layers of Silica Gel H, 0.25 mm thick, with hexane/diethyl ether (95:5) as the developing solvent. Aliquots of 5-10 mg were applied to 20 × 20 cm layers, which had been prewashed with diethyl ether. The component lipid classes were detected by spraying the edges of the plates with 0.1%

ethanolic 2',7'-dichlorofluorescein solution and eluted from the adsorbent with water-saturated diethyl ether.

The ratios of lipid classes were determined by gas liquid chromatography (GLC) with some modification of the procedures reported by Christie et al. (30). A definite amount of heptadecyl heptadecanoate was added as internal standard to aliquots of the extracted lipid classes. In addition, the fractions containing wax esters and steryl esters were reduced with $LiAlH_4$ in diethyl ether (31). Each of the lipid fractions was analyzed using a Perkin-Elmer F-22 gas liquid chromatograph equipped with 2 flame ionization detectors and 2 glass columns (1.8 m × 3 mm) packed with 3% OV-101 on Gas-Chrom Q, 100-120 mesh (Applied Science Laboratories, Inc.). Nitrogen was used as carrier gas at a flow rate of 40 ml/min. The temperature was programmed from 180-250 C, 4 C/min, and then kept at 250 C for 70 min. Peak areas were measured with an Autolab System IV B Spectra Physics Reporting Integrator.

Analysis of Lipid Fractions

Hydrocarbon fractions were further resolved by argentation chromatography (32) on layers of Silica Gel G, 0.25 mm thick, containing 5% silver nitrate with hexane as the developing solvent. The fractions consisting of alkanes were eluted from the adsorbent with water-saturated diethyl ether and the components were identified by combined gas liquid chromatography-mass spectrometry (GLC-MS) using an LKB 9000 instrument equipped with a glass column (1 m × 2 mm) packed with 3.8% UC W 98 on Supelcoport, 80-100 mesh (Supelco, Inc., Bellefonte, PA). The flow rate of helium was 30 ml/min and the temperature was programmed from 250-300 C at 2 C/min. The separator and ion source both were maintained at 300 C. The mass spectrometer was operated at 70 eV and a trap current of 60 μA. The percentage composition of alkanes was determined by GLC on 3% OV-101, as just described.

Fractions consisting of wax esters and steryl esters were resolved on layers of magnesium oxide, 0.3 mm thick, with hexane/diethyl ether/ethyl acetate (75:25:1) as the developing solvent (33). The fractions of wax esters and steryl esters were each eluted from the adsorbent with water-saturated diethyl ether, and then transmethylated (34). The resulting mixtures of methyl esters and long chain primary alcohols as well as methyl esters and sterols, respectively, were separated on layers of Silica Gel H, 0.25 mm thick, with hexane/diethyl ether (80:20) as the developing solvent. The fractions were eluted from the adsorbent with diethyl ether saturated with water.

Fractions consisting of primary alcohols were acetylated in acetic anhydride/pyridine (5:1) (35), and the resulting acetates were purified on layers of Silica Gel H, 0.25 mm thick, with hexane/diethyl ether (80:20) as the developing solvent.The acetates were recovered from the adsorbent by elution with water-saturated diethyl ether and analyzed by a Varian Aerograph 2700 gas liquid chromatograph attached to a Varian MAT CH 7 mass spectrometer. The column (1.8 m × 2 mm) was packed with 3% Silar-5 CP on Gas-Chrom Q, 100-120 mesh (Applied Science Laboratories, Inc.); the helium flow rate was 30 ml/min at 245 C. Both the separator and ion source were maintained at 250 C. The mass spectrometer was operated at 70 eV and a trap current of 300 μA. The acetates were identified by comparison with the retention times and mass sepctra of reference mixtures, which were prepared from BC Mix branched chain fatty acid methyl esters (Applied Science Laboratories, Inc.) via reduction with $LiAlH_4$ and subsequent acetylation. The percentage composition of primary alcohols was determined by GLC on 3% OV-101, as already described.

Samples of methyl esters, before and after catalytic hydrogenation over platinium (35), were analyzed by GLC on glass columns (1.8 m × 3 mm) packed with 10% EGSS-X on Gas-Chrom P, 100-120 mesh (Applied Science Laboratories, Inc.) or 3% OV-101 (as described) at 200 and 250 C, respectively.

The composition of fractions consisting of ketones or secondary alcohols was determined directly by GLC on 3% OV-101, using long chain ketones (ICN Pharmaceuticals) or their $LiAlH_4$ reduction products, respectively, as reference compounds.

Aldehyde fractions containing trace amounts of ethyl esters were resolved by chromatography on layers of Silica Gel H, 0.25 mm thick, with hexane/diethyl ether (95:5) as the developing solvent. The chromatoplates were developed twice, and the 2 lipid fractions were eluted from the adsorbent with water-saturated diethyl ether. The aldehydes were reduced with $LiAlH_4$ to primary alcohols, which were subsequently acetylated and analyzed as already described. Fractions of ethyl esters were characterized by GLC, both directly and after transmethylation, as described for the analysis of methyl esters.

Fractions consisting of free fatty acids were esterified with diazomethane (36), and the resulting methyl esters were purified on layers of Silica Gel H, 0.25 mm thick, with hexane/

diethyl ether (80:20) as the developing solvent. The methyl esters were recovered from the adsorbent by elution with water-saturated diethyl ether and analyzed by GLC as already described.

RESULTS AND DISCUSSION

Cabbage seedpod weevils produce only one generation/year and depend on immature seed pods of rape for oviposition. The cuticular lipids obtained from 4000 sexually mature beetles (6 g) and from 1000 g of immature seed pods of host plants amounted to 20 mg and 500 mg, respectively. The quantitative distribution of lipid classes in cuticular lipids of *C. assimilis* and *B. napus* seed pods, given in Table I, shows that hydrocarbons were most abundant in both lipids, and that various esters, ketones, aldehydes and alcohols as well as free fatty acids occurred in minor proportions only.

The hydrocarbon fraction of cabbage seedpod weevils was found to contain only traces of olefins. The saturated hydrocarbon fraction of *C. assimilis* revealed the presence of homologous series of *n*-alkanes, iso-alkanes,

TABLE I

Composition (%) of Cuticular Lipids of Cabbage Seedpod Weevils *(C. assimilis)* and of Host Plant *(B. napus)* Seed Pods

Lipid classes	*C. assimilis*	*B. napus*
Hydrocarbons	77	63
Wax esters and steryl esters	6	9[a]
Ketones	5	15
Aldehydes	2[b]	3
Unidentified	1	
Secondary alcohols	2	8
Primary alcohols	tr[c]	2
Fatty acids	7	tr[c]

[a]Wax esters exclusively.

[b]Inclusive traces of ethyl esters.

[c]tr = Trace < 0.5%.

TABLE II

Composition (%) of Hydrocarbons from Cuticular Lipid Fractions of Cabbage Seedpod Weevils *(C. assimilis)* and of Host Plant *(B. napus)* Seed Pods

Number of carbon atoms	*C. assimilis*[a]				*B. napus*[b]
	n-Alkanes	iso-Alkanes	X-Methylalkanes[c]	X,Y-Dimethylalkanes[c]	*n*-Alkanes
23	tr[d]				
25	1				tr
26	tr	(tr)[d]			tr
27	1	tr			2
28	tr	(tr)	tr		> 1
29	14	5	tr		95
30	tr	tr	7		< 1
31	tr	3	tr	tr	1
32			4	tr	
33			(tr)	18	
34			tr	1	
35				40	
36				< 1	
37				3	
Total	> 16[e]	< 9	< 12	< 63	100

[a]Trace amounts of unsaturated hydrocarbons were not analyzed.

[b]Hydrocarbons other than *n*-alkanes were not detected.

[c]X = Position of methyl branches predominantly at C_{11}, C_{13} or C_{15}; Y = position of second methyl group predominantly at C_{X+4}.

[d]tr = Trace < 0.5%; (tr) = trace <0.1%.

[e]Proportion of *n*-alkanes as percentage of total hydrocarbons was subject to strong seasonal variations from 12-45%.

internally branched monomethylalkanes and internally branched dimethylalkanes, ranging from 23 to 37 carbon atoms (Table II). The iso-branched hydrocarbons were identified by means of relatively large (M-43) fragments in the mass spectra in conjunction with the absence of (M-29) fragments; the internally branched hydrocarbons were identified on the basis of characteristic mass ratios of even- and odd-numbered fragments resulting from the branching position (37,38).

The major components of terminally branched hydrocarbons were iso-alkanes with 29 and 31 carbon atoms, whereas anteiso-alkanes could not be detected (Table II). The principal constituents of internally branched monomethylalkanes had 30 and 32 carbon atoms, whereas in dimethylalkanes components with 33, 35 and 37 carbon atoms predominated. All internally branched hydrocarbons consisted of isomeric mixtures with one methyl branch predominantly in 11-, 13- or 15-positions or 2 methyl groups in 11, 15-, 13, 17- or 15, 19-positions, respectively.

It should be noted that a variety of mono- and dimethylbranched alkanes, detected in cuticular lipids of cabbage seedpod weevils, have been documented in the literature as components of close-range sex pheromones of house flies (39) and stable flies (14,16). Homologous cuticular or synthetic hydrocarbons of different chain lengths have been reported as contact mating pheromones of the tsetse fly (21) and the biting midge (22).

The *n*-alkane fractions from cuticular lipids of both *C. assimils* and seed pods of *B. napus* contained *n*-nonacosane as the predominant component (Table II). In *B. napus* seed pods *n*-nonacosane constituted as much as 95% of the total hydrocarbons, and hydrocarbons other than *n*-alkanes were not detected at all. Epicuticular leaf waxes from *B. napus* also have been found to contain *n*-nonacosane as the prominent component (40). In *C. assimilis*, the relative proportion of *n*-nonacosane of the total hydrocarbons was found to increase from 12% in April to up to 45% by the end of June. Although the reason for these seasonal variations of *n*-nonacosane was not investigated, it is interesting to note that in grasshoppers the incorporation of dietary *n*-alkanes into cuticular lipids has been reported (41,42).

In cabbage seedpod weevils, the cuticular lipids contained 6% of various classes of esters and 7% of free fatty acids, whereas those in host plant seed pods contained 9% of esters and 2% of free primary alcohols (Table I). The ester fraction in *C. assimilis* was found to be composed of equal proportions of wax esters and steryl esters, whereas in *B. napus* only wax esters were present. The qualitative composition of esterified primary alcohols of wax esters and that of unesterified primary alcohols revealed a fairly close resemblance between beetles and seed pods (Table III). In each case, mixtures of *n*, iso and anteiso homologs were found, mainly in the range of 26-30 carbon atoms, and the composition of mixtures was qualitatively similar to corresponding fractions from normal and mutant forms of *B. napus* leaves (40).

TABLE III

Composition (%) of Esterified Primary Alcohols of Wax Esters and Free Primary Alcohols from Cuticular Lipid Fractions of Cabbage Seedpod Weevils *(C. assimilis)* and of Host Plant *(B. napus)* Seed Pods

Number of carbon atoms	*C. assimilis*			*B. napus*					
	Esterified primary alcohols			Esterified primary alcohols			Free primary alcohols		
	n	iso	anteiso	*n*	iso	anteiso	*n*	iso	anteiso
14	tr[a]			1			tr		
16	3			tr			tr		
18	1	(tr)[a]		tr	(tr)		tr		
19						(tr)			
20	1	tr		tr	tr		tr	(tr)	
21			(tr)			(tr)			
22	7	1		< 1	tr		1	(tr)	
23			3			< 1			< 1
24	3	1		2	tr		3	tr	
25			2			2			< 1
26	8	10		16	2		28	1	
27			32	tr		54	1		35
28	4	5		6	2		15	1	
29	1		8	tr		12	1		7
30	< 1	9		tr	< 1		1	4	
Total	29	26	45	26	5	69	> 50	> 6	< 44

[a]tr = Trace < 0.5%; (tr) = trace < 0.1%.

TABLE IV

Composition (%) of Esterified and Free Fatty Acids from Cuticular Lipid Fractions of Cabbage Seedpod Weevils *(C. assimilis)* and of Host Plant *(B. napus)* Seed Pods

Number of carbon atoms: number of double bonds	*C. assimilis*								*B. napus*		
	Esterified fatty acids of wax esters			Esterified fatty acids of steryl esters			Esterified fatty acids of ethyl esters	Free fatty acids	Esterified fatty acids of wax esters		
	n	iso	anteiso	*n*	iso	anteiso	*n*	*n*	*n*	iso	anteiso
12:0	1			1					1		
14:0	11			6			< 1	1	2		
15:0	tr[a]		tr	tr		tr			< 1		tr
16:0	15	tr		11	tr		11	16	13	1	
16:1	2			3			3	6			
17:0	tr		4	1		3			tr		18
18:0	5	1		4	1		2	5	28	3	
18:1	26			30			37	38			
18:2	9			13			19	13			
18:3	12			19			27	19			
19:0			3			2					18
20:0	3	1		1	tr			tr	5	tr	
21:0			tr			tr					1
22:0	<1	tr		2	tr			tr	1	tr	
22:1	2			1				< 1			
23:0			tr			tr					1
24:0	1	tr		1					1	tr	
25:0			1								1
26:0	1	tr		tr					1	tr	
27:0											1
28:0									< 1	< 1	
29:0											tr
30:0									tr		
31:0											tr
Total	89	> 2	> 8	> 93	> 1	> 5	100	> 99	54	5	> 40

[a]tr = Trace < 0.5%.

TABLE V

Composition (%) of Aldehydes, Ketones and Secondary Alcohols from Cuticular Lipid Fractions of Cabbage Seedpod Weevils *(C. assimilis)* and of Host Plant *(B. napus)* Seed Pods

Number of carbon atoms	*C. assimilis*					*B. napus*				
	Aldehydes			Ketones	Secondary alcohols	Aldehydes			Ketones	Secondary alcohols
	n	iso	anteiso	*n*	*n*	*n*	iso	anteiso	*n*	*n*
14	tr[a]					(tr)[a]				
16	1					1				
18	1					1				
20	1					(tr)				
22	12					1				
24	5					< 1				
25	< 1		2			tr		1		
26	18	tr		> 1	2	5	(tr)		tr	tr
27	1		4		< 1	1		2	tr	1
28	16	tr		tr	< 1	28	tr		< 1	> 1
29	3		2	98	95	3		1	98	96
30	30	< 1		(tr)	1	53	tr		(tr)	> 1
31				(tr)					< 1	(tr)
32	2					1				
Total	91	1	8	> 99	> 99	95	tr	4	> 99	> 99

[a]tr = Trace < 0.5%; (tr) = trace < 0.1%.

Although quantitative differences of iso- and *n*-alcohols were observed, it appears likely that primary alcohols in cuticular lipids of cabbage seedpod weevils are of host plant origin.

The major portion of esterified primary alcohols from cuticular lipids of cabbage seedpod weevils was composed of anteiso homologs (Table III), whereas the terminally branched hydrocarbons contained exclusively iso-alkanes (Table II). Thus, in the beetle, the biosynthesis of cuticular hydrocarbons obviously does not correlate with the presence or absence of primary alcohols.

Table IV shows that the esterified and free fatty acids from cuticular lipid fractions of cabbage seedpod weevils and of host plant seed pods occurred predominantly in the range of 14-22 carbon atoms. Wax esters of *B. napus* seed pods, in conformity with wax esters of leaves (40), contained only saturated esterified fatty acids that were composed of roughly equal proportions of branched and unbranched components. On the other hand, esterified fatty acids of wax esters and steryl esters from *C. assimilis* consisted of major proportions of saturated as well as unsaturated *n*-compounds, whereas iso- and anteiso structures were present in minor proportions only. Nevertheless the predominant esterified branched fatty acids of the host plant wax esters, anteiso with 17 and 19 carbon atoms, may be contributing to the esterified anteiso fatty acids of the insect wax and steryl esters. The unesterified fatty acids from cuticular lipids of *C. assimilis* were completely devoid of branched components and were mainly composed of stearic, oleic, linoleic and linolenic acids. A similar composition of esterified fatty acids was also found in the ethyl ester fraction from cuticular lipids of cabbage seedpod weevils (Table IV). In this context, it is interesting to note that ethyl palmitate, stearate, oleate and linoleate have been claimed to be biologically active constituents of the assembling scent from the Khapra beetle (43,44).

The composition of aldehydes, ketones and secondary alcohols from cuticular lipid fractions of cabbage seedpod weevils and host plant seed pods is given in Table V. All components were found to be saturated. The aldehyde fractions of both cuticular lipids consisted of more than 90% unbranched compounds with 26, 28 and 30 carbon atoms as the major constituents. Minor proportions of anteiso-aldehydes had odd carbon numbers; iso-aldehydes were found in traces only. All fractions of ketones and secondary alcohols contained unbranched compounds exclusively, and, as in corresponding fractions from normal and

mutant forms of *B. napus* leaves (40), *n*-nonacosanone or secondary *n*-nonacosanol, respectively, were the prominent components. The ratios and composition of corresponding fractions of aldehydes, ketones and secondary alcohols from cuticular lipids of *C. assimilis* and *B. napus* indicate close resemblance (Tables I and V). Because these components are quite common in plants and rare in insects, the small amounts of aldehydes, ketones and secondary alcohols on the beetle suggest that they could be of host plant origin.

The occurrence of nonacosanone and homologous long chain ketones in the cuticular lipids of cabbage seedpod weevils (Table V) is notable with regard to the fact that ketones of such chain lengths, isolated from cuticular waxes of female house flies and of female German cockroaches, have been evaluated or identified, respectively, as close-range sex pheromones (11,23,24).

Considering the discovery of potential pheromones as constituents of the cuticular lipids investigated, tests on biological activity are in progress.

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METHODS

Quantitative Analyses of Hydroxystearate Isomers from Hydroperoxides by High Pressure Liquid Chromatography of Autoxidized and Photosensitized-oxidized Fatty Esters[1]

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ABSTRACT

A high pressure liquid chromatography (HPLC) method is described for the determination of the isomeric hydroxystearates from hydroperoxides of oxidized fatty esters. The samples are hydrogenated and the mixtures of hydroxystearates are concentrated by partial removal of unoxidized esters and complete removal of polar materials. Isomeric hydroxystearates are then separated on a porous microparticulate adsorption (10 μ) column and elution with 0.25% isopropyl alcohol in *n*-hexane is monitored at 212 nm. The 8-OH, 9-OH, 10-OH, 11-OH and 16-OH isomers were completely separated, but the 12-OH, 13-OH and 15-OH were only partly resolved by HPLC. The relative percentages of isomeric hydroxy esters were analyzed quantitatively by a computer integration method. Accuracy of the method was checked with known mixtures of synthetic hydroxystearates. The isomeric hydroperoxide composition of oxidized methyl oleate, linoleate, linolenate and soybean methyl esters determined by HPLC were in good agreement with previous analyses by gas chromatography-mass spectrometry.

INTRODUCTION

Recently, renewed interest in the problems of lipid oxidation has resulted in improved methodology for the analysis of fatty acid hydroperoxides. Gas chromatography-mass spectrometry (GC-MS) was applied to the study of hydroperoxides from oxidized methyl oleate, linoleate, linolenate and their mixtures by quantitative analysis of the silylated hydroxystearate derivatives (1-3). GC analysis was used to determine allylic hydroxy esters as trimethylsilyl (TMS) derivatives in mixtures of oxidized oleate and linoleate (4). Capillary GC was also used to separate the TMS ethers of 9- to 14-hydroxystearates (5). On one hand, high pressure liquid chromatography (HPLC) was used to analyze conjugated allylic hydroxy esters from oxidized linoleate (6) and linolenate (7) by ultraviolet (UV) detection at 234 nm for conjugated diene absorption. On the other hand, HPLC was used to analyze the hydroxystearate derivatives from oleate hydroperoxides by UV detection at 212 nm for ester absorption using 0.75% ethanol in hexane as solvent system (8).

In this paper, the Chan and Levett HPLC method (8) has been extended and improved to allow quantitative analysis of isomeric hydroxystearate composition from different hydroperoxides formed by both autoxidation and photosensitized oxidation of methyl oleate, linoleate, linolenate and soybean esters. The quantitative accuracy was checked with known mixtures of synthetic hydroxystearates and the results were compared with previous analyses by GC-MS (1-3).

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METHODOLOGY

HPLC Equipment and Conditions

A Water's HPLC system was used with a loop injector and a column (0.39 x 30 cm) packed with 10-μ Porasil (Waters Associates, Milford, MA); flow: 1.5 ml/min; solvent: 0.25% isopropanol in hexane; sample size: 1.0-2.4 mg hydroxystearate dissolved in mobile phase; detector: variable wavelength UV (Schoeffel Instruments, Westwood, NJ) set at 212 nm. Detector output was quantitated by computer as area percentage along with absolute retention time. The same synthetic methyl 9-, 10, 13-, and 16-hydroxy esters were used as before (1-3). The methyl 15- and 17-hydroxystearates were synthesized by literature methods (9). Autoxidation and hydrogenation procedures were also the same. Photosensitized oxidations were described previously (10).

Sample Concentration for HPLC

Hydroxystearates from hydrogenated-oxidized samples were concentrated by partial removal of nonoxidized components and complete removal of polar compounds to avoid column contamination. This concentration procedure was accomplished either with the disposable short pipette procedure described previously (11) or with silica Sep Pak (Waters Associates). The Sep Pak procedure consisted of using a 200-mg sample dissolved in 1 ml hexane, eluting most of the methyl stearate with 10 ml hexane, and then eluting all the hydroxystearates with 5 ml 1:1 diethyl ether/hexane. More polar compounds remained on the Sep Pak. All separations were monitored by thin layer chromatography (TLC) (60:40 diethyl ether/hexane) using synthetic hydroxystearate standards (1-3).

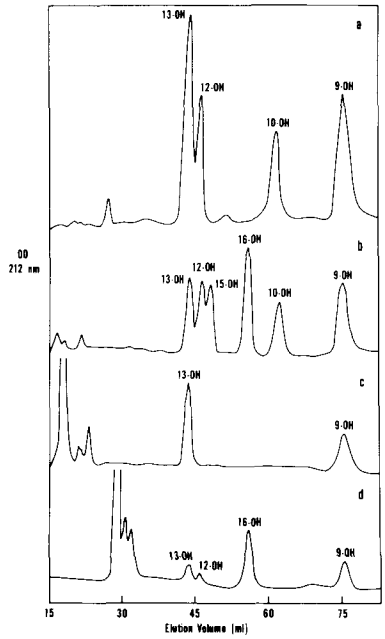

FIG. 1. HPLC chromatograms of hydroxystearates for the analysis of hydroperoxides; column: 0.39 X 30 cm 10 μ Porasil; flow; 1.5 ml/min; solvent: 0.25% isopropanol in hexane; sample size: 1.0-2.4 mg hydroxystearate in mobile phase; detector: variable wavelength ultraviolet at 212 nm. (a) Hydrogenated photosensitized-oxidized linoleate (PV = 1124); (b) hydrogenated photosensitized-oxidized linolenate (PV = 1566); (c) hydrogenated autoxidized linoleate (PV = 115); and (d) hydrogenated autoxidized linolenate (PV = 132).

RESULTS AND DISCUSSION

Typical separations of isomeric hydroxystearates from oxidized fatty esters on a μ-Porasil column are presented in Figure 1. Peak identification is based on retention of synthetic hydroxystearates. With the 0.25% isopropanol

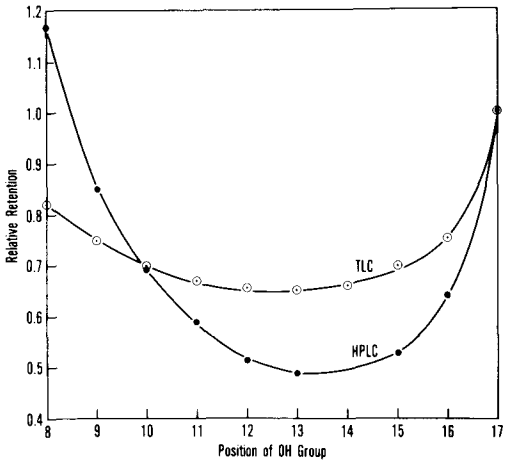

FIG. 2. Retention of hydroxystearates by HPLC and TLC. See Figure 1 for HPLC conditions. TLC data obtained from Morris and Wharry (12); conditions—plate: Silica Gel G; solvent: diethyl ether/light petroleum (1:1). Retention is expressed relative to methyl 17-hydroxystearate: for HPLC = corrected retention volume of OH stearate/corrected retention volume of 17-OH stearate; for TLC = distance from origin of OH stearate/distance from origin of 17-OH stearate.

TABLE I

HPLC Analysis of Synthetic Hydroxystearates

Mixture[a]	Relative percent						Standard deviation[b]	Standard error[b]
	9-OH	10-OH	12-OH	13-OH	15-OH	16-OH		
1	51.7	48.3					1.0	0.6
2	29.8	21.2	25.0	24.0			1.2	0.6
3	17.7	29.8	32.5	19.6			0.4	0.2
4	16.8	16.7	17.5	14.4	16.4	18.1	0.8	0.5
5	26.7	12.8	10.7	9.6	11.8	28.2	0.9	0.5

[a]Weight % composition of synthetic mixtures: 1: 50.0 each; 2: 30.0, 20.0, 20.0, 30.0; 3: 20.0, 30.0, 30.0, 20.0; 4: 16.7 each; and 5: 30.0, 10.0, 10,0, 10.0, 10.0, 30.0.

[b]By analysis of variance (14).

TABLE II

HPLC[a] and GC-MS[b] Analyses of Hydroxystearates in Hydrogenated Autoxidized and *Photosensitized-oxidized Fatty Esters*

Esters Oxidation[c], PV	Analysis method	Relative percent 8-OH	9-OH	10-OH	11-OH	12-OH	13-OH	15-OH	16-OH	Standard deviation[d]
Oleate										
Auto-, 1232	HPLC	25	25	22	28					1.1
	GC-MS	26	24	24	26					
Photo-, 1727	HPLC		51	49						2.1
	GC-MS		48	52						
Linoleate										
Auto-, 115	HPLC		49				51			0.7
	GC-MS		48				52			
Photo-, 1124	HPLC		32	16		17	35			0.5
	GC-MS		32	17		17	35			
Linolenate										
Auto-, 710	HPLC		37			8	10		45	1.5
	GC-MS		34			10	12		44	
Auto-, 1315	HPLC		28			9	11		51	1.5
	GC-MS		32			8	10		50	
Photo-, 1566	HPLC		20	13		14	15	12	26	1.1
	GC-MS		23	13		12	14	13	25	
Soybean										
Auto-, 517	HPLC	3	45	1	2	2	39		7	1.0
	GC-MS	2	43	2	1	3	40		9	

[a]See conditions in Methodology section in text.

[b]GC-MS conditions (1).

[c]Autoxidation (1) of oleate at 80 C, linoleate 30 C, linolenate 40 C (PV 710) and 25 C (PV 1315); photosensitized-oxidations at 0 C (5).

[d]By analysis of variance (14).

in hexane solvent system, elutions relative to methyl 17-hydroxystearate (1.0) used as internal standard, are as follows: 13-OH, 0.49; 12-OH, 0.52; 15-OH, 0.53; 11-OH, 0.59; 16-OH, 0.64; 10-OH, 0.69; 9-OH, 0.85; and 8-OH, 1.17. The 8-OH, 9-OH, 10-OH, 11-OH and 16-OH isomers are completely separated, but the 12-OH, 13-OH and 15-OH are only partly resolved by HPLC. When the 0.75% ethanol in hexane solvent system of Chan and Levett (8) was tried with our column, we failed to resolve the 12-, 13-, and 15-hydroxystearates, a separation that is required for the analysis of photosensitized-oxidized linoleate and linolenate.

The isomeric hydroxysterates show a similar change in elution order by HPLC as previously reported by Morris and Wharry using TLC (12). For comparison with HPLC, the TLC data were expressed as the reciprocal of the relative retention measured as the relative distance of spots from the origin (Fig. 2). There is a change in retention from the 17- to the 13-hydroxystearates and then another change in retention down to 8-hydroxystearate. The steeper change in retention between 8- and 13- and between 15- and 17-OH isomers observed by HPLC affords better separation than by TLC. Critical pairs that are more difficult to separate by TLC than by HPLC include 11- and 14-OH, 10- and 15-OH, 9- and 16-OH isomers.

The relative percentage of hydroxystearate isomers was analyzed quantitatively by integrating peak areas with a computer method involving first and second derivatives of the raw data determined when peaks start and stop (13). Precision and accuracy of the HPLC method were evaluated by analyzing known mixtures of synthetic hydroxystearates. The average of replicate determinations was calculated by computer and standard deviation and error were calculated by analysis of variance (14). The accuracy of the analyses is reflected by the standard deviation from known composition, which varied from 0.4 to 1.2 (Table I). Standard error ranged from 0.2 to 0.6. The standard deviations are in the same range as previously found for GC-MS analyses of synthetic hydroxystearate mixtures (1-3).

HPLC was compared with the GC-MS method for the determination of isomeric hydroperoxides (1-3,10,11). Table II shows good agreement between these 2 methods in the analyses of hydroperoxides from different autoxidized and photosensitized-oxidized fatty esters. Replicate results computed by analysis of variance (14) gave standard deviations ranging from 0.5 to 2.1. The HPLC method described in this paper is therefore complementary to GC-MS for the analysis of hydroperoxides from methyl oleate, linoleate, linolenate and soybean esters.

The agreement between results of the HPLC and GC-MS methods provides further confidence in this new methodology. The isomeric hydroperoxide composition of autoxidized and photosensitized-oxidized oleate, linoleate, linolenate and soybean esters has now been fully confirmed by 2 independent HPLC and GC-MS methods. The results with linolenate hydroperoxides obtained by autoxidation also confirm previous analysis using a third entirely different approach based on cleavage analysis (15). These quantitative studies of isomeric hydroperoxides form the basis for clarifying the mechanism of free radical autoxidation and photosensitized oxidation of unsaturated fatty esters (16,17).

ACKNOWLEDGMENTS

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The Removal of Peroxides from Phosphatidylcholine Preparations

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ABSTRACT

A major portion of the peroxides from egg yolk phosphatidylcholine preparations can be removed by filtration through Sephadex LH-20, by repetitive suspending of phosphatidylcholine preparations in water, precipitation by acetone and brief incubation of the lipid with superoxide dismutase.

INTRODUCTION

Peroxides and other products of oxidation are usually present in phosphatidylcholine preparations, although the peroxide content depends on various factors such as the method of purification, type of storage and admixture of certain organic compounds or heavy metals. It is necessary, therefore, to assess the peroxide content of phospholipids at different stages of purification in order to obtain homogeneous, peroxide-free phospholipid preparations. The thiobarbituric acid (TBA) test and the ratio $A_{233\ nm}/A_{215\ nm}$ in optical spectra of phosphatidylcholines are sensitive criteria for such assessment (1,2).

The complete removal of peroxides from phosphatidylcholine preparations and from other phospholipids by a rapid and simple method still is often desirable. We report here how this can be accomplished.

MATERIALS AND METHODS

Phosphatidylcholine from egg yolks was prepared essentially according to Bangham et al. (3) by chromatography on aluminum oxide and silicic acid columns. The phospholipid purification was completed within 48 hr at 4 C. The phosphatidylcholine thus obtained was homogeneous as judged by thin layer chromatography (TLC) on silica gel plates ("Silufol," Czechoslovakia) using chloroform/methanol/ammonia (230:90:15, v/v) or chloroform/methanol/water (65:25:4, v/v) as developing systems. Fractions were detected using Dragendorf's solution (4) for choline-containing compounds and a phospholipid stain reagent (5). Only a single spot was detected by either of the methods just described or by using sulfuric acid spray on chromatograms of freshly prepared phosphatidylcholine. The peroxide content in phosphatidylcholine preparations was determined from their spectral ratio, A_{233} nm/A_{215} nm, in ethanol solution as well as by the TBA assay (1). Superoxide dismutase (SOD) was obtained from bovine erythrocytes according to the McCord and Fridovich method (6). The concentration of the enzyme was determined from molar extinction of 300 M^{-1} cm^{-1} for the absorption at 680 nm. Optical spectra were recorded at 20 C on Specord (GDR) or Beckman model 26 instruments using 10 mm cells. All solvents were distilled twice immediately before use. Sephadex LH-20 and Sepharose 4B were obtained from Pharmacia. All other chemicals of "Puriss" grade were manufactured in the USSR.

RESULTS AND DISCUSSION

In accordance with numerous earlier studies, we observed that freshly prepared phosphatidylcholine stored in the presence of oxygen for several weeks increased in peroxide content as determined by both the methods already described. Thus, the ratio $A_{233\ nm}/A_{215\ nm}$ was increased from 0.13, which is typical for freshly prepared phosphatidylcholine, to 0.50 or higher for the preparations stored for 3 wk. According to these criteria, the peroxide content was raised during storage from 0.065% to 2.5%. These data correlate reasonably well with results of the TBA test.

In an attempt to decrease the peroxide content or phosphatidylcholine preparations, the phospholipids were filtered through a Sephadex LH-20 column. For this purpose, the lipids were dissolved in ethanol and administered to a Sephadex LH-20 column that had been preconditioned with ethanol. Gel filtration of the phosphatidylcholine resulted in the partial removal of peroxides. However, the resolution of phospholipids and of their peroxides was not completely effective, probably because of the small differences in molecular weights. Figure 1 shows the typical elution pattern of phosphatidylcholine and of the fraction corresponding to peroxides and compares the spectra of both fractions.

In the following series of experiments, the

effect of SOD on the peroxide content was tested. This enzyme is known to be very active in the dismutation of superoxide anion-radicals, $O_2^{\cdot-}$, and in the destruction of H_2O_2 and of organic peroxides (7-10). It was found that the incubation of phosphatidylcholine preparations with SOD results in a further decrease of the peroxide content in the phospholipid. In these experiments, phospholipid preparations were suspended in water at concentrations of 10-30 mg/ml and 10^{-6}-10^{-7} M of the enzyme was added to the suspension. The suspension was incubated at room temperature for 1-30 min, then 3 vol of acetone were added and the precipitate formed was collected by centrifugation and dissolved in ethanol. The effect of SOD on the peroxide content in phosphatidylcholine preparations is shown in Figure 2. The data indicate that the incubation of the phospholipid with SOD results in the almost complete destruction of peroxide.

We also found that when preparations with high peroxide content were suspended in water and then sedimented by addition of acetone, the residue had remarkably lower peroxide content than the untreated preparations. In experiments we undertook in this series, typically 20-30-mg portions of the phospholipid were suspended in 3-5 ml of distilled water, and 3 times this vol of acetone was added at room temperature. The precipitate was collected by centrifugation at 3000 x g for 5 min and dissolved in ethanol to compare its spectrum with that of the initial preparation of phosphatidylcholine and to determine the peroxide content by TBA assay. After this treatment, the peroxide content of phosphatidylcholine was significantly decreased. When

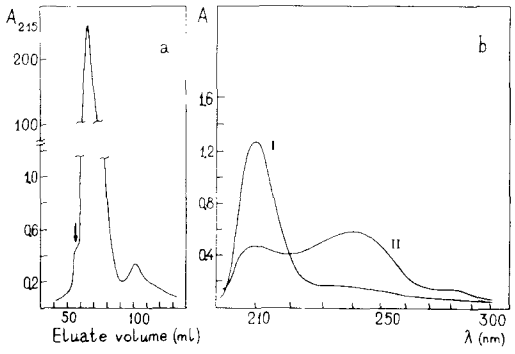

FIG. 1. (a) Elution pattern of phosphatidylcholine gel filtration through Sephadex LH-20. The lipid (100 mg) was dissolved in ethanol (1.5 ml) and administered onto the column (2 cm x 45 cm). The shoulder of the peroxide fraction is shown by an arrow; (b) optical spectra of central (I) and peroxide (II) fractions.

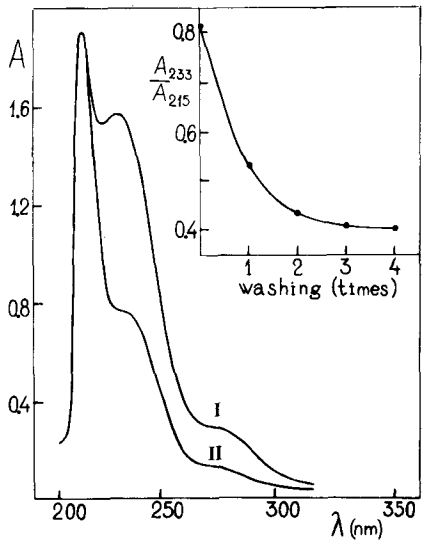

FIG. 2. The effect of SOD on spectral purity index of the phosphatidylcholine; (a) initial spectrum (after 4 treatments with water and acetone, see b in Figure 3); (b) spectrum of the same preparation after additional incubation with 3 x 10^{-7} M SOD for 5 min. The insert shows the dependence of the spectral index on SOD concentration.

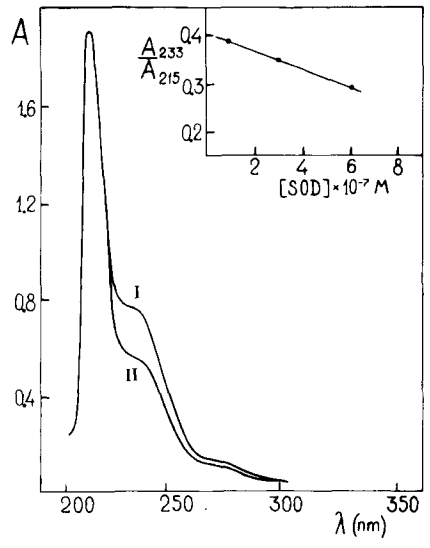

FIG. 3. The effect of the water/acetone washing procedure on the spectral purity index of phosphatidylcholine; (a) spectrum of an initial peroxide-containing preparation; (b) spectrum of the same preparation after treatments with water and acetone. The insert shows the dependence of the spectral index on the number of washings.

TABLE I

The Effect of Repeated Washing on the Peroxide Content of a Phosphatidylcholine Preparation

	Initial content of peroxides	No. of washings			
		1	2	3	4
According to spectral criteria	2.5%	1.8%	1.6%	1.4%	1.1%
According to TBA test	0.6%	0.5%	0.4%	0.35%	0.3%

the treatment was repeated 3-4 times, the peroxide content was lowered by 50-60% as shown in Figure 3 and Table I. It is important to note that the washing of the lipid with water or with acetone alone did not lead to the removal of peroxides and that precipitation is required.

The 3 procedures described for the removal of overall products of oxidation from phosphatidylcholine can be used separately or in combination. We were able to prepare rather stable phosphatidylcholine liposomes with 0.5% peroxide content from the initial phosphatidylcholine preparation containing 4% peroxides using only the treatment of the initial preparation by SOD. The water suspension of the lipid was first of all incubated twice with 10^{-6} M enzyme and then the lipid obtained was used for the preparation of liposomes by the Papahadjopoulos and Miller method (11). We also observed that the addition of 10^{-7} M SOD to liposomes has a remarkably stabilizing effect on the liposome structures. Traces of SOD can be removed by gel filtration of the suspended liposomes through a Sepharose 4B column.

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A Convenient Spectrophotometric Assay for Phospholipase D Using *p*-Nitrophenyl-phosphocholine As Substrate[1]

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ABSTRACT

A simple assay for plant phospholipase D is described. The enzyme hydrolyzes *p*-nitrophenyl-phosphocholine to *p*-nitrophenyl phosphate, which in the presence of an acid phosphatase generates *p*-nitrophenol (a chromogenic compound). Thus, *p*-nitrophenylphosphocholine can be used for assay of phospholipase D in sources that do not also contain high levels of phospholipase C.

INTRODUCTION

Phospholipase D (EC 3.1.1.4) is generally found in the tissues of higher plants and has both hydrolase and transferase activity, with a broad specificity towards phosphatides:

$$\text{Phosphatidylcholine} + H_2O \xrightarrow{\text{Phospholipase D}} \text{Phosphatidic acid} + \text{choline}$$

$$\text{Phosphatidylcholine} + \text{ROH} \xrightarrow{\text{Phospholipase D}} \text{Phosphatidyl-OR} + \text{choline}$$

The hydrolase activity usually predominates unless a high concentration of alcohol is present (1). Both activities are generally assayed by following the liberation of choline from an emulsion of phosphatidylcholine (PC). This involves separation of choline from the reaction mixture and subsequent quantification of the isolated choline either chemically or radiochemically (2). The available assay can thus be somewhat cumbersome and may require special experience and may depend, for example, on the quality of the PC dispersion (3,4).

We wish to report here a convenient assay for phospholipase D which is an extension of an assay already available for phospholipase C (5). Phospholipase C is reported to hydrolyze *p*-nitrophenylphosphocholine (NPPC) to liberate *p*-nitrophenol which can be measured spectrophotometrically. We have found that the same substrate NPPC is hydrolyzed by phospholipase D apparently to give *p*-nitrophenyl-phosphate since the subsequent addition of acid phosphatase liberates *p*-nitrophenol. We have used this coupled assay to detect and follow the purification of phospholipase D from plant source.

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MATERIALS AND METHODS

Phospholipase D from peanut (type II), phospholipase D from cabbage (type I), phosphodiesterase I (*crotalus adamanteus* venom, type II) and NPPC were obtained from Sigma Chemical Company. Acid phosphatase (human prostatic) was purchased from the Calbiochem-Behring Corp. All other chemicals were of analytical grade.

Assay Procedure

The extent of hydrolysis of NPPC was monitored by measuring the absorption of liberated *p*-nitrophenol at 400 nm. NPPC (25 μmol in 0.5 ml 50 mM acetate buffer, pH 5.6) containing 50 mM Ca^{++} was mixed with 0.4 units of acid phosphatase in the same buffer and varying amounts of phospholipase D were added together with the appropriate amount of acetate buffer to give a total vol of 0.9 ml. After incubation at 37 C, 100 μl of 0.05 M NaOH was added to terminate the reaction and the absorbance was read at 400 nm against a blank from which phospholipase D had been omitted.

RESULTS AND DISCUSSION

The time course for NPPC hydrolysis with different concentrations of phospholipase D given in Figure 1(a) shows that the reaction is linear with time. Like Kurioka and Matsuda (5), we also have used NPPC concentrations which are below the estimated K_m value of 0.05 M. This may account for the nonlinearity observed with respect to enzyme concentration above ca. 12 units of enzyme (Fig. 1[b]. Thus, under the conditions reported here, the practical range of enzyme concentration assay is limited to 0-12 units. The fact that a 5-fold increase in phosphatase concentration did not alter the results in Figure 1 confirms the con-

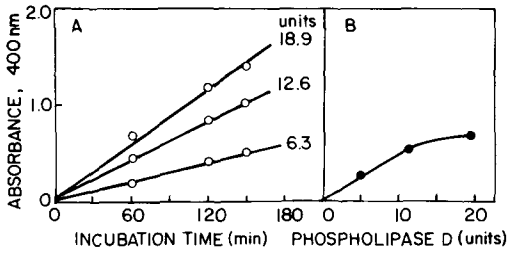

FIG. 1(a) Time course for NPPC hydrolysis at 37 C by phospholipase D. The amount of phospholipase D in the reaction mixture is given in activity units next to each line; (b) dependence of the rate of hydrolysis at 37 C of NPPC on the concentration of phospholipase D. The experimental conditions for both a and b are given in the text. The results shown were all obtained with the commercial preparation of peanut phospholipase D. Identical results were obtained with both crude and purified preparations of peanut and cabbage phospholipase D. Phospholipase D alone or phosphatase alone gave no measurable color production. The amount of enzyme added is indicated in units of phosphatidylcholine (PC) activity. One unit will liberate 1 μmol of choline from PC/hr under the conditions used; thus, the reactive rates with the natural substrate and with NPPC are 25:1 using 4 times higher concentration of NPPC (25 mM) than that used in the standard assay with PC (6 mM).

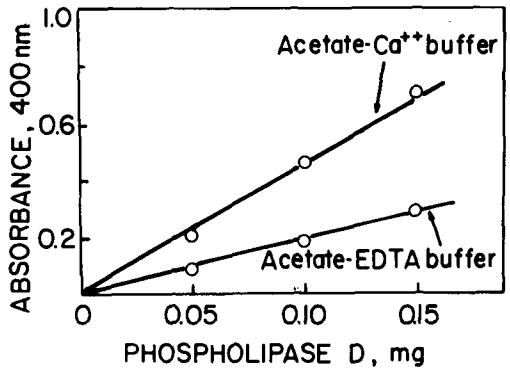

FIG. 2. Inhibition of peanut phospholipase D activity by EDTA. The experimental conditions are those described in the text with incubation for 1 hr at 37 C. In the EDTA-buffer, Ca^{++} was replaced by 0.1 M EDTA. Increasing the EDTA concentration to 1 M did not give further inhibition. Enzyme activity: 50 units/mg of protein.

clusion that the observed hydrolysis rate is a function of phospholipase D concentration under the conditions used. Kurioka and Matsuda (5) have mentioned that the presence of 60% sorbitol accelerates NPPC hydrolysis by phospholipase C. In the case of phospholipase D, we did not observe any increase in the rate of hydrolysis when sorbitol was added.

With PC as substrate, phospholipase D is reported to require Ca^{++} for optimal activity. It also has been mentioned that EDTA inhibits the activity at a concentration lower than that required for chelation of Ca^{++}, suggesting that the enzyme might require the presence of another unknown trace metal (4). Our observations with NPPC as substrate (Fig. 2) agree with those findings. When the reaction with NPPC is carried out in absence of Ca^{++} and with 1 mM or 0.1 M EDTA incorporated in the buffer, the enzyme activity is significantly lowered and not abolished. It thus appears that although Ca^{++} activates the enzyme, is not an absolute requirement when NPPC is used as substrate. This conclusion is consistent with the suggestion that a major role of Ca^{++} in the phospholipase-D-catalyzed hydrolysis of the natural substrate is in forming the proper dispersion of the substrate (4).

In using this assay, we were concerned about the possible interference of nonspecific phosphodiesterases. Some phosphodiesterases like bovine spleen diesterase do not catalyze the hydrolysis of NPPC (5). However, we have found that venom phosphodiesterase I has substantial activity with this substrate (Fig. 3). A convenient way to check for general phosphodiesterase activity is to use bis-(*p*-nitrophenyl) phosphate, which is a good substrate for diesterases in general, but which is not a substrate for phospholipase D. In the case of partially purified phospholipase D preparations from cabbage and peanuts, which give good levels of activity with NPPC as substrate, no activity was observed when bis-(*p*-nitrophenyl)-phosphate was used. Thus, the activity measured in these preparations can be concluded to represent phospholipase D activity. It may be useful to note in this connection that the venom phosphodiesterase activity can be significantly lowered in the presence of 10^{-3} M Cu^{++} (Fig. 3) (6), which does not affect the phospholipase D activity. Most of the phosphodiesterases have a specific metal requirement or are inhibited by certain metal ions and in general it should be possible to use such properties to distinguish between general phosphodiesterase activity and combined activity of phospholipase D and endogenous phosphatase (6).

A final consideration for the validity of this assay for phospholipase D is the possible ambiguity in distinguishing between high levels of phospholipase C activity and combined activity of phospholipase D and endogenous phosphatase. Since we do not know of systems where the 2 enzymes coexist, this problem is unlikely to arise. In any case, specific stimulation of phospholipase C activity by sorbitol may be used to resolve this potential ambiguity.

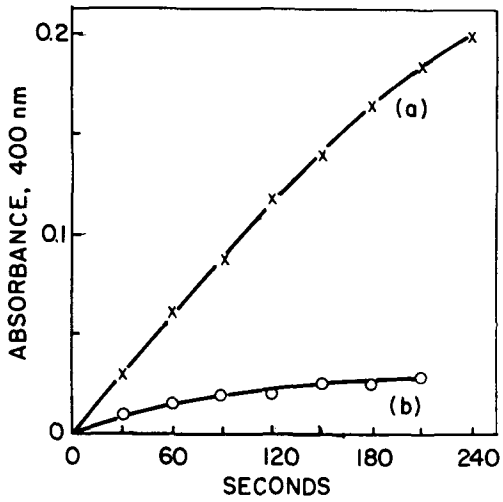

FIG. 3. The effect of Cu^{++} on the rate of NPPC hydrolysis catalyzed by venom phosphodiesterase I. NPPC, 50 μmol in 1.0 ml of 50 mM acetate buffer, pH 4.5, was incubated with 1 mg of diesterase at 37 (a) in the absence of Cu^{++} and (b) in the presence of 10^{-3} M Cu^{++}.

Since our assays were conducted under conditions very similar to those used by Kurioka and Matsuda (5) for phospholipase C, it is possible to estimate that comparable quantities of phospholipases C and D have widely different activities toward NPPC. Thus, it appears that phospholipase D is about 200 times more active than phospholipase C in the absence of sorbitol and 20 times more active in the presence of 60% sorbitol, and that it may be possible to assay phospholipase D even in the presence of equal quantities of phospholipase C in the absence of sorbitol.

As is the case for most artificial substrates, the use of NPPC will probably not yield any unique insight regarding the in vivo function of phospholipase D. In our hands it has, however, provided a fast, easy and reproducible assay by which phospholipase D purification from plant sources can be monitored spectrophotometrically with a readily available substrate.

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COMMUNICATIONS

Species Variation in Serum Levels of Prostaglandins and Their Precursor Acids

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ABSTRACT

Levels of prostaglandin (PG) precursor acids, and PGE_2, $PGF_{2\alpha}$ and TXB_2 in sera of adult animals (5/species) were determined by gas chromatography and radioimmunoassay, respectively. The level of arachidonic acid in horse serum lipids was the lowest (0.61 ± 0.03%), and that in dog serum lipids was the highest (18.1 ± 1.8%). Bovine serum lipids contained considerable amounts of 20:3ω6 (precursor of monoene PG) and 20:5ω3 (precursor of triene PG) in addition to arachidonic acid. Thromboxane B_2 was not the major species of endoperoxide metabolite synthesized by platelets from arachidonic acid in male ruminants and pig. The concentration of TXB_2 in the serum of the lactating cow was more than 50 times greater than that of ovariectomized cows or of bulls. Although TXB_2 was the major species of endoperoxide metabolite synthesized by human platelets, its serum concentration was much lower than that of nonruminant animals except the pig. These results showed that there were considerable variations in levels of PG and their precursors among various species of animals. The species variation in PG and TXB_2 concentrations was not simply attributed to the differences in platelet concentration blood.

INTRODUCTION

Most animal cells are capable of synthesizing prostaglandins (PG). Amounts and kinds of PG synthesized vary with types of tissues and species of animals (1,2). The capability of platelets to synthesize PG and/or thromboxanes has been demonstrated in humans (3), rats (4,5), horses (6) and monkeys (7). However, comparative capacities of platelets from different species of animals have not been reported. Circulating blood does not contain detectable amounts of PG because they are rapidly metabolized in major organs and excreted. Serum concentrations of PG indicate amounts of PG synthesized by platelets during clotting of the blood withdrawn from circulation. Human erythrocytes cannot synthesize PG and neither erythrocytes nor plasma can metabolize PG synthesized by platelets (8-10). Therefore, measuring serum PG may be more quantitative than measuring PG in other tissue homogenates possessing enzymes metabolizing the PG.

The PG precursor acids—eicosatrienoic acid (20:3ω6), arachidonic acid (20:4ω6) and eicosapentaenoic acid (20:5ω3)—in animal tissues can be derived from linoleic acid or linolenic acid by desaturation and elongation. For animals which are incapable of desaturating linoleic acid or linolenic acid, the direct PG precursors have to be derived from dietary sources. As an example, the cat is not able to desaturate linoleic acid to arachidonic acid (11). Since most animal foods contain the direct PG precursor acids, ominivores and carnivores can obtain the precursor acids directly from their diets regardless of their abilities to desaturate linoleic acid or linolenic acid. However, in herbivores, the precursor acids have to be synthesized from linoleic acid or linolenic acid in their tissues. Therefore, composition of the precursor acids in tissue lipids in herbivores can provide information about types of PG and thromboxanes (i.e., monoene, diene or triene PG) which can be synthesized in the tissue. This study was to determine relative concentrations of PG and their precursor acids among various species of animals.

MATERIALS AND METHODS

Venous blood samples were collected from 5 adult male animals. The animals were randomly selected from research farms belonging to the Department of Animal Science, Department of Dairy Science, Department of Poultry Science and the School of Veterinary Medicine, Louisiana State University. Each species of animal was kept on the same dietary regimen. Blood samples for humans were obtained from

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volunteer graduate students (2 males and 4 females). The blood samples were incubated at 37 C for 1 hr to optimize PG production (12). Sera were stored at -20 C until assayed.

Serum samples were extracted according to a modification of the Gréen et al. method (13). Three ml of acetone cooled to -20 C was added to 1ml serum and the mixture was shaken for 1 min. Three ml of petroleum ether (bp 30-60 C) was added, vortexed for 1 min and centrifuged briefly. The petroleum ether layer containing neutral lipids and most of the acetone was removed. This step was repeated once. The pH of aqueous phase was adjusted to 3.5 with 2% formic acid and the mixture was extracted twice with 5 ml diethyl ether. The diethyl ether layer was transferred to a flask and evaporated under nitrogen. The trace of formic acid was eliminated through additional amounts of diethyl ether and repeated evaporation. The residue was dissolved in phosphate-buffered saline (0.14 M NaCl, 0.01 M $NaPO_4$, 0.1% gelatin, pH 7.0). The recovery of tritiated thromboxane B_2 (120 ci/mnol, New England Nuclear, Boston, MA) added into rat serum was 87 ± 0.9%. The loss of radioactivity in the petroleum ether phase was less than 2%.

Concentrations of PG in the extracted samples were determined by radioimmunoassay. Antibodies for PGE_2 and $PGF_{2\alpha}$ used in this study were prepared previously and assessment of the validity of the assay system, characterization of antibodies and analysis of the data using a radioimmunoassay computer program (14) were described in previous reports (5,12,15). The preparation of TXB_2 antibody was also described in a previous report (15). Tritiated PGE_2 (130 ci/mmol) and tritiated $PGF_{2\alpha}$ (178 ci/mmol), purchased from New England Nuclear, were used without further purification. Fatty acid composition assays of serum lipids with emphasis on PG precursor acids were carried out as previously reported (5,12).

RESULTS AND DISCUSSION

Plants do not contain arachidonic acid; therefore, the presence of arachidonic acid in tissue lipids of herbivores reflects the capability to desaturate and elongate linoleic acid. Among herbivores studied in this report, rats showed the highest concentration of arachidonic acid in serum lipids as shown in Table I. Presence of Δ^5 and Δ^6 desaturases in the rat were well documented (16,17). Serum lipids of rats also contained a considerable amount of 20:5ω3 (the precursor of triene PG) which is derived from 18:3ω3. In the dog, an omnivore, arachi-

TABLE I

Fatty Acid Composition of Serum Lipids of Various Species of Animals[a]

Fatty acids	Bull	Cat	Chicken	Cow	Dog	Horse	Human	Lactating cow	Monkey	Pig	Rabbit	Rat	Sheep
16:0	14.5 ± 0.6	15.3 ± 0.6	20.8 ± 0.4	14.4 ± 0.8	15.9 ± 0.5	15.0 ± 0.4	21.7 ± 0.6	12.4 ± 0.9	16.9 ± 0.4	16.1 ± 0.8	23.5 ± 0.6	21.3 ± 0.5	17.5 ± 0.6
16:1	4.7 ± 0.5	4.2 ± 0.5	3.1 ± 0.2	3.2 ± 0.6	2.7 ± 0.06	1.8 ± 0.3	3.2 ± 0.4	3.3 ± 0.3	1.9 ± 0.2	2.4 ± 0.1	3.8 ± 0.2	3.2 ± 0.2	5.0 ± 0.3
18:0	17.2 ± 1.2	16.0 ± 0.9	15.6 ± 0.6	20.8 ± 0.4	20.5 ± 1.0	12.7 ± 0.4	7.3 ± 0.3	15.4 ± 1.0	12.1 ± 0.7	11.6 ± 0.3	9.2 ± 0.3	9.7 ± 0.2	15.6 ± 0.9
T18:1ω9		0.9 ± 0.01	1.0 ± 0.05	2.3 ± 0.05									
C18:1ω9	13.8 ± 0.8	29.7 ± 1.0	17.9 ± 0.4	11.4 ± 1.2	17.6 ± 0.8	13.0 ± 0.4	22.7 ± 1.0	10.4 ± 1.1	14.2 ± 0.6	23.2 ± 0.7	17.1 ± 1.1	14.7 ± 0.8	22.3 ± 1.6
18:2ω6	35.5 ± 1.4	29.2 ± 0.6	22.4 ± 0.8	34.3 ± 1.6	22.9 ± 1.7	54.5 ± 1.3	32.7 ± 1.4	48.2 ± 2.4		37.1 ± 1.5	34.1 ± 0.7	24.6 ± 0.6	22.9 ± 1.3
18:3ω6	0.6 ± 0.06	0.3 ± 0.06	0.3 ± 0.05	1.3 ± 0.09			0.3 ± 0.03	1.0 ± 0.06	0.2 ± 0.03		0.4 ± 0.08	0.5 ± 0.01	0.4 ± 0.01
18:3ω3	6.5 ± 0.3	0.8 ± 0.1	0.5 ± 0.04	5.2 ± 0.3		1.0 ± 0.2	0.4 ± 0.1	3.0 ± 0.2	0.3 ± 0.08	0.5 ± 0.03	6.9 ± 0.8	0.4 ± 0.1	6.3 ± 1.2
20:1ω9	0.2 ± 0.03												
20:2ω6	0.7 ± 0.03	0.3 ± 0.01	0.4 ± 0.03	0.4 ± 0.02	0.3 ± 0.09	0.3 ± 0.1	0.3 ± 0.06	0.2 ± 0.03	0.4 ± 0.03				
20:3ω6	0.7 ± 0.09	0.3 ± 0.1	1.0 ± 0.09	2.6 ± 0.1	1.1 ± 0.2		1.0 ± 0.1	1.6 ± 0.1	1.2 ± 0.2	0.3 ± 0.05	0.3 ± 0.07	0.2 ± 0.02	
20:3ω3	0.7 ± 0.07	0.1 ± 0.01	0.5 ± 0.06	1.4 ± 0.03		0.8 ± 0.1		0.2 ± 0.06	0.3 ± 0.08	0.7 ± 0.09	1.8 ± 0.2	0.6 ± 0.03	0.8 ± 0.6
20:4ω6	4.3 ± 0.4	2.1 ± 0.2	9.7 ± 0.2	3.3 ± 0.3	18.1 ± 1.8	0.6 ± 0.03	8.0 ± 0.9	2.6 ± 0.14	5.6 ± 0.5	7.5 ± 0.5	1.8 ± 0.2	17.1 ± 1.4	5.3 ± 0.6
20:5ω3	0.5 ± 0.04	0.6 ± 0.1	1.0 ± 0.07	1.5 ± 0.2			0.4 ± 0.1	0.4 ± 0.1			0.2 ± 0.04	2.7 ± 0.2	1.1 ± 0.2
22:1ω9			0.2 ± 0.01										
22:4ω6	0.2 ± 0.03		0.5 ± 0.03		1.0 ± 0.3		0.2 ± 0.03	0.2 ± 0.03	0.6 ± 0.06	0.3 ± 0.06		0.2 ± 0.07	
22:5ω6	0.3 ± 0.03		2.4 ± 0.3				0.2 ± 0.03		0.4 ± 0.06		0.3 ± 0.07	0.2 ± 0.01	0.8 ± 0.2
22:5ω3	0.8 ± 0.05	0.2 ± 0.01	0.7 ± 0.08				0.2 ± 0.06	0.4 ± 0.08	0.3 ± 0.09	0.3 ± 0.05	0.9 ± 0.3	1.6 ± 0.9	1.3 ± 0.2
22:6ω3	0.7 ± 0.07	0.9 ± 0.06	4.4 ± 0.1				1.6 ± 0.2		0.2 ± 0.02			3.3 ± 0.09	0.8 ± 0.05
Unknown[b]				0.6 ± 0.04				0.2 ± 0.02					

[a]Values are mean of 5 samples ± SEM (weight percent).

[b]Based on its equivalent chain length, it was tentatively identified as 20:4ω3.

donic acid could be derived from the diet or from linoleic acid by desaturation and elongation. Polyunsaturated fatty acids of the ω3 family were not found in dog serum. Major emphasis in PG research has been focused on diene PG derived from arachidonic acid because it is the major precursor acid found in tissue lipids of humans and many other species of animals. However, bovine serum lipids contained considerable amounts of 20:3ω6 (precursor of monoene PG) and 20:5ω3 in addition to arachidonic acid. It was shown that bovine erythrocytes do not contain arachidonic acid (18) and the major PG precursor acid in bovine seminal vesicles was 20:3ω6 (1). It is important to determine relative amounts of monoene and triene PG compared to diene PG synthesized in bovine tissues since different PG can exert different physiological responses and potencies. The percentage of arachidonic acid in horse serum lipids was the lowest, whereas that of linoleic acid was the highest among animals studied, implying that horses may have limited capacity to desaturate linoleic acid to arachidonic acid. Triene PG precursor acids (20:5ω3) was undetected in horse serum lipids whereas 18:3ω3 was present at a 1% level. Chicken and cat sera showed considerable amounts of fatty acids of the ω3 family. These fatty acids might be derived from their diets containing fish products. It was shown that the cat is incapable of desaturating linoleic acid to arachidonic acid (11); therefore, arachidonic acid in cat serum lipids also might be derived from its diet. Except in humans, dogs, rats and pigs, 20:3ω3 was present in serum lipids of other animals (Silar-10C column used in this study gave a baseline separation for 20:3ω3 and 20:4ω6 methyl ester standards). Serum lipids of most herbivores contained relatively large amounts of 18:3ω3, which might be derived from chloroplast lipids. One of the major unsaturated fatty acids in chloroplast lipids is known to be linolenic acid (19). Bovine serum lipids contained an unknown fatty acid which was tentatively identified as 20:4ω3 based on its equivalent chain length.

Human platelets can synthesize PG E, F and D groups, and particularly thromboxane as a major product from arachidonic acid. However, it is unknown whether platelets from other species of animals can also synthesize thromboxane as a major product from arachidonic acid. The data indicated (Table II) that thromboxane B_2 was not the major product of arachidonic acid in ruminants (except lactating cows) and pigs, whereas it was the major product of arachidonic acid in other species of animals. The concentration of TXB_2 in ruminants and pigs was not significantly different from that of $PGF_{2\alpha}$ or PGE_2. In the chicken, the concentration of TXB_2 was about 150 times higher than that of $PGF_{2\alpha}$. Although TXB_2 was the major PG in human serum, its concentration was much lower than in sera of nonruminant animals except for the pig. The concentration of TXB_2 in serum of the lactating cow was more than 50 times greater than that of bulls or ovariectomized cows. It is unknown what causes this drastic increase in PG synthesis by platelets of lactating cows. Analysis of serum samples obtained from another set of animals (lactating cows and nonlactating but nonovariectomized cows) indicated that increased synthesis of TXB_2 in lactating cows may not result from lactation itself but from having an esterous cycle (data not shown). Systematic experiments are being

TABLE II

Concentrations of Serum PG in Various Species of Animals (ng/ml)[a]

Animals	PGE_2	$PGF_{2\alpha}$	TXB_2
Bull	0.68 ± 0.20	3.28 ± 1.32	2.54 ± 0.39
Cat	1.64 ± 0.61	4.73 ± 1.37	127.10 ± 13.08
Chicken	1.57 ± 0.32	2.66 ± 0.69	396.38 ± 99.98
Cow[b]	0.69 ± 0.09	1.70 ± 0.23	1.69 ± 0.47
Dog	67.28 ± 15.25	25.54 ± 3.04	782.81 ± 166.45
Horse	0.5 ± 0.07	0.56 ± 0.05	26.41 ± 6.13
Human	0.97 ± 0.26	1.41 ± 0.29	24.44 ± 6.37
Lactating cow	2.04 ± 0.27	6.67 ± 0.64	139.15 ± 11.65
Monkey	6.00 ± 1.84	12.64 ± 2.11	365.72 ± 61.20
Pig	1.81 ± 0.68	4.47 ± 0.33	7.72 ± 1.78
Rabbit	4.92 ± 0.68	8.54 ± 2.11	290.67 ± 38.48
Rat	6.78 ± 1.51	11.01 ± 2.20	97.59 ± 21.62
Sheep	0.34 ± 0.07	2.02 ± 0.36	1.66 ± 0.03

[a]Values are mean ± SEM of 5 samples.

[b]Ovariectomized cow.

carried out to elucidate this finding. Species variation in the concentration of $PGF_{2\alpha}$ did not coincide with that of TXB_2; therefore, species variation in PG concentrations was not simply attributed to the difference in platelet concentration in blood.

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Major Unsaturated Cuticular Hydrocarbons of the Field Crickets, *Gryllus pennsylvanicus* and *Nemobius fasciatus*[1]

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ABSTRACT

Adult male field crickets, *Gryllus pennsylvanicus* Burmeister, have more unsaturated cuticular hydrocarbons than adult females, and each has more unsaturates than last-instar male and female nymphs. The 2 most abundant unsaturated hydrocarbons from each adult were (*Z*)-7-nonacosene and (*Z,Z*)-6,22-nonacosadiene. Last-instar male nymphal field crickets, *Nemobius fasciatus* (De Geer), were almost devoid of cuticular alkenes compared to last instar female nymphs and adult males and females. The major unsaturated hydrocarbon from each was (*Z,Z*)-6,9-pentacosadiene.

INTRODUCTION

The cuticular saturated hydrocarbons from mixed sexes of field crickets have been identified as homologous series of *n*-alkanes and 2-methylalkanes in *Nemobius fasciatus* (De Geer) along with internally branched monomethyl- and dimethylalkanes in *Gryllus pennsylvanicus* Burmeister (1); however, no mention of unsaturated hydrocarbons was made.

The presence of cuticular alkenes was a possibility since similar materials were found in the house cricket, *Acheta domesticus* (L.) (2). We therefore investigated the cuticular lipids of the field crickets for alkenes that might be present in only one sex.

EXPERIMENTAL PROCEDURES

G. pennsylvanicus and *N. fasciatus* adults and nymphs were collected in Baltimore County, MD. Adult males and females on a diet of Southern States 350 chicken crumb were held in separate cages for 3 weeks before being used; last-instar nymphs were used soon after collection.

Cuticular lipids of male and female nymphs and adults were extracted by immersing the insects in hexane. The lipids were fractionated in a manner similar to that described by Warthen and Uebel (2) and examined by gas liquid chromatography (GLC) on a 91 × 0.2 cm id glass column packed with 2% OV-101 on Chromosorb W-HP.

Hydrocarbons were separated from nonhydrocarbons on Florisil (60-100 mesh) by eluting with hexane and diethyl ether, respectively. The hydrocarbons containing saturates and unsaturates were analyzed by GLC and then ozonized (3). Comparison of the GLC analysis of the ozonized-hydrolyzed mixture with that of the original mixture revealed which components were unsaturated.

The hydrocarbons of the adult males and females were separated into alkanes, monoenes and dienes on 20% silver nitrate-impregnated Florisil by eluting with increasing concentrations of diethyl ether in hexane.

The monoenes and dienes were dissolved in hexane and hydrogenated while stirring over platinum oxide. Mass spectra of these hydrocarbons were obtained from a Hewlett-Packard 5992A GC/MS equipped with a glass column packed with 2% OV-101.

The positions of the double bonds were located in the following manner: the 29-carbon monoenes and dienes and the 25-carbon diene were isolated by preparative GLC and ozonized-hydrolyzed (3); the resulting aldehydes were identified by comparing retention times to those of authentic standards.

RESULTS

G. pennsylvanicus

Table I shows the amount of cuticular lipid and percentage hydrocarbon for *G. pennsylvanicus*. GLC analysis of the nonhydrocarbon fractions revealed numerous minor nonhydrocarbons with the same retention times (R_t) as many of the hydrocarbons.

Last-instar male nymphs and female nymphs contained unsaturated hydrocarbons as 2 GLC peaks at 29- and 30-carbon numbers (R_t) and 1 GLC peak at 29-carbon number (R_t), respectively. Unsaturated hydrocarbons were found among the cuticular lipids of both the adult males and females as 3 GLC peaks; the largest amount of unsaturates for both sexes occurred

[1] Orthoptera: Gryllidae.

TABLE I

Major Cuticular Unsaturated Hydrocarbons in *G. pennsylvanicus* and *N. fasciatus*

	G. pennsylvanicus				*N. fasciatus*			
	Adults		Nymphs		Adults		Nymphs	
	♂	♀	♂	♀	♂	♀	♂	♀
Cuticular lipid (mg/insect)	0.62	0.77	0.39	0.41	0.20	0.20	0.12	0.14
Hydrocarbon (%)	81	64	72	69	100	100	100	100
Unsaturated hydrocarbon (%)	–	–	28	31	45	57	9	59
Abundant monoene	–[a]	–[a]	–	–	–	–	–	–
Abundant diene	–[b]	–[b]	–	–	–[c]	–[c]	–	–[c]

[a](*Z*)-7-Nonacosene.
[b](*Z,Z*)-6,22-Nonacosadiene.
[c](*Z,Z*)-6,9-Pentacosadiene.

just prior to the R_t of the 29-carbon *n*-alkane standard (Fig. 1). The other 2 occurred just prior to the R_t for 27- and 31-carbon numbers.

Silver nitrate-impregnated Florisil chromatography revealed that the hydrocarbons from adult males contained 47% alkanes, 24% monoenes and 29% dienes; the hydrocarbons from adult females contained 82% alkanes, 11% monoenes and 7% dienes. The most abundant materials in the monoenes and dienes from both adult males and females were analyzed by GC-MS. A 29-carbon monoene and a 29-carbon diene were found in each sex. When each of these materials was hydrogenated, *n*-nonacosane was obtained.

Silver nitrate thin layer chromatography (TLC) of the monoenes from adult males and females with authentic *cis* and *trans* standards (2) showed the monoenes to have only the *cis* configuration. Ozonolysis-hydrolysis of the double bond of the 29-carbon monoenes produced heptanal and docosanal in nearly the same quantities for both males and females. Thus, the most abundant monoene for both the males and females was (*Z*)-7-nonacosene.

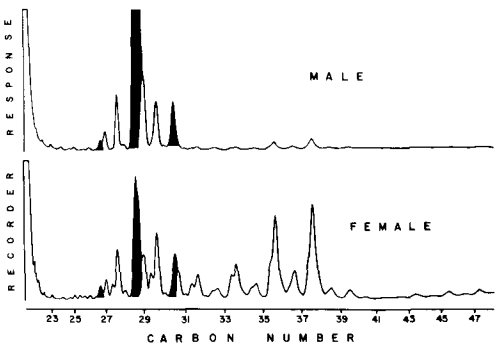

FIG. 1. Gas chromatograms of the cuticular hydrocarbons washed from adult male and female *G. pennsylvanicus*. Shaded peaks are unsaturated hydrocarbons. The column was 2% OV-101 (91 × 0.2 cm id glass) operated at 185 C for 2 min and then programmed at 2 C/min to 305 C with a flow rate of 30 ml/min.

The diene fractions from adult males and females showed no IR absorption indicative of double bonds in the *trans* configuration or in the terminal position. Ozonolysis-hydrolysis of the 29-carbon dienes from both the males and females yielded hexanal and heptanal as the 2 most abundant aldehydes and hexadecanedial as the most abundant dialdehyde. Further confirmation of this fragmentation was obtained by a partial ozonolysis-hydrolysis of the nonacosadienes; docosenal and tricosenal were the major unsaturated aldehydes obtained from both sexes. Thus, the predominant diene was (*Z,Z*)-6,22-nonacosadiene.

N. fasciatus

Table I also shows the amount of cuticular lipid and percentage hydrocarbon for *N. fasciatus*. A major peak of unsaturated hydrocarbon was present in the adult males and females and the female nymphs (Fig. 2) but present in only a minor amount in male nymphs. The mass spectrum of this peak revealed a molecular ion of m/e 348 and the mass spectrum of its hydrogenated product had a molecular ion of m/e 352. The mass spectrum and retention time of this product were identical to those of *n*-pentacosane.

Silver nitrate TLC of this unsaturated hydrocarbon revealed that it was a diene, and the IR spectrum indicated the presence of *cis* double bonds.

Ozonolysis-hydrolysis of the diene produced hexadecanal and hexanal, whereas partial ozon-

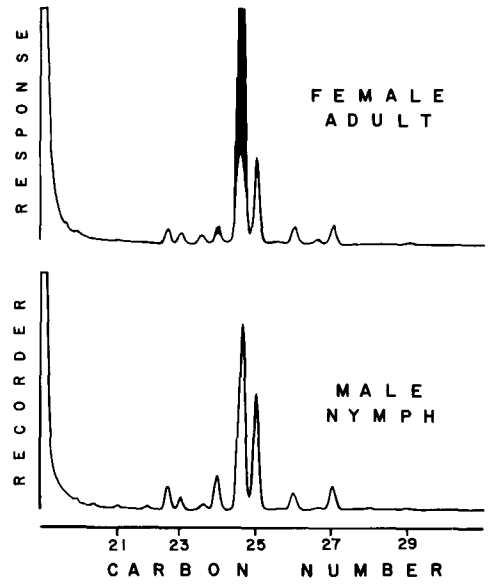

FIG. 2. Gas chromatograms of the cuticular hydrocarbons washed from adult females and last-instar male *N. fasciatus*. Shaded peaks are unsaturated hydrocarbons. The column was 2% OV-101 (91 × 0.2 cm id glass) operated at 155 C for 2 min then programmed at 2 C/min to 220 C with a flow rate of 30 ml/min.

olysis-hydrolysis produced nonenal and nonadecenal. The aldehydes were identified by mass spectral fragmentation and/or GC retention time. The major cuticular diene of male and female *N. fasciatus* was (*Z,Z*)-6,9-pentacosadiene.

DISCUSSION

Alkenes and alkadienes are present in the cuticular hydrocarbons of several insects (4-12). The major hydrocarbon component of *Periplaneta americana* (L.) is (*Z,Z*)-6,9-heptacosadiene (13,14). The sex attractant of *Musca domestica* L. present in the cuticular hydrocarbons is (*Z*)-9-tricosene (15-17). Beeswax also contains a number of alkenes of primarily the (*Z*)-configuration (18-20).

The 25-carbon alkadiene present in *N. fasciatus* falls into the 6,9-diene class present in *P. americana*. However, *G. pennsylvanicus* has a 29-carbon alkadiene with double bonds in the 6,22-position rather than the 6,9. Double bonds at the seventh carbon atom have also been reported (21) for cuticular monoenes in the little housefly, *Fannia canicularis* (L.). *G. pennsylvanicus* also has a 7-monoene of 29 carbons.

The major unsaturated cuticular hydrocarbons in *A. domesticus* are 37- and 39-carbons as opposed to the shorter ones of *G. pennsylvanicus* and *N. fasciatus*. *G. pennsylvanicus* adult males contained more unsaturated cuticular hydrocarbons than adult females,as did *A. domesticus* adult males. However, the adults and female nymphs of *N. fasciatus* contained equal amounts of unsaturated cuticular hydrocarbons; the male nymphs contained a very small amount of unsaturated hydrocarbon. The biological function of these cuticular components remains to be discovered.

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Effect of Dietary Fat on the Fluidity of Platelet Membranes

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ABSTRACT

Dietary fat type was reflected in the phospholipid fatty acid composition of the plasma membrane of rabbit platelets and apparently controlled the fluidity of these membranes. Rabbits were maintained for 6 months on diets that varied in stearic and polyunsaturated fatty acids and thus had different potentials for thrombosis. Microviscosities at 37 C, calculated from the anisotropy of fluorescence from the probe 1,6-diphenyl-1,3,5-hexatriene, were 3.5, 3.4, 2.8 and 2.2 poise for platelet membranes isolated from rabbits whose only source of dietary fat was cocoa butter, milkfat, coconut oil, or corn oil, respectively. The relative fluidities of the membrane isolates were correlated with the polyunsaturated fatty acid contents of the membrane phospholipids.

INTRODUCTION

Diets that are high in long chain, saturated fatty acids, particularly stearic have been implicated as predisposing animals to thrombosis (1,2). Yet, our present knowledge of the role of nutrition in hemostasis and thrombosis is limited. Research pertaining to the interrelationships between chemical structure and physical surface properties of the platelet should help define the role of ingested fat by increasing our understanding of the molecular basis of platelet physiology. We now report the effects of dietary fats, which vary in their contents of stearic acid, polyunsaturated fatty acids and short chain fatty acids, upon the chemical composition and fluidity of the plasma membranes of rabbit platelets, as determined by fluorescence polarization.

MATERIALS AND METHODS

Animals and Diets

Male New Zealand white rabbits weighing 1.8-2.0 kg were purchased (Camm Research Institute) and fed commercial stock diet (Purina) ad libitum until they weighed 3.6-4.0 kg. The animals were randomly divided into 4 groups of 5 rabbits and fed experimental diets containing different natural fats.

The Gaman et al. semipurified diet (3) minus corn oil was used as a basal diet. Pelleted diets consisting of the basal diet plus 5% cocoa butter, milkfat, coconut oil, or corn oil were prepared commercially (Teklad Test Diets) to our specifications. Fatty acid contents of the prepared diets (Table I) determined by gas chromatography (GC) were typical of these fats. The rabbits were allowed free access to food and water during the 6-month test period.

Membrane Isolation

Blood samples (~30 ml) were drawn via cardiac puncture into plastic syringes containing Na_2EDTA in saline as an anticoagulant. Platelets were isolated by differential centrifugation (4) and washed by repeated centrifugation in Gaintner's (5) phosphate buffer, pH 7.5, with added Na_2EDTA (1 mg/ml). Platelets from rabbits consuming each diet were pooled, dispersed in washing buffer and lysed by the Barber and Jamieson glycerol lysis technique

TABLE I

Fatty Acid Composition of Diets (mol %)

Diet	Cocoa butter	Milkfat	Coconut oil	Corn oil
Acid				
10:0	–	0.71	1.70	–
12:0	–	3.68	50.25	–
14:0	0.43	13.42	21.71	–
16:0	27.39	37.28	11.36	13.59
16:1	0.39	2.89	–	–
18:0	30.92	11.63	2.57	2.20
18:1	34.31	26.46	8.77	26.58
18:2	4.94	3.06	3.64	55.45
18:3	0.35	0.88	–	1.61
20:0	1.27	–	–	0.57

(6). The lysed cell suspension was centrifuged through a 27% sucrose cushion (ρ=1.101 g/cm^3) in a swinging bucket rotor at 86,000 × g for 3 hr to isolate the plasma membrane fraction at the buffer-sucrose interface. The membrane isolate was pelleted without further subfractionation in an angle head rotor at 143,000 × g for 1 hr and dispersed in Gaintner's phosphate buffer for further study.

Fluidity Measurements

Fluidity of the membrane lipid phase was determined from measurements of depolarization of fluorescence from the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) by the Shinitzky and coworkers methods (7-9). For probe incorporation, 2 × 10^{-3} M DPH in tetrahydrofuran was diluted 500- to 1000-fold in aqueous suspensions of platelet membrane isolates and incubated with agitation at 35-37 C for 2 hr.

Steady state fluorescence polarization intensity was measured with an Aminco-Bowman spectrophotofluorometer equipped with Glan-Thompson prism polarizers. DPH was excited at 366 nm and the fluorescence at 450 nm was detected through a Wratten 2A cutoff filter for wavelengths shorter than 415 nm. Light scattering errors were minimized by diluting the membrane suspensions until the polarization or anisotropy remained constant. Fluorescence data were taken as a function of temperature from 45 to 0 C at intervals of 3-6 degrees.

Microviscosities were calculated from the anisotropy data with the approximation:

$$\eta = 2.4r/(0.362-r)$$

by Shinitzky and Barenholz (9) of the Perrin equation for the anisotropy, r, of DPH fluorescence. The measured anisotropies were obtained from the intensities of emission polarized parallel and perpendicular to the polarized excitation using standard formulas (10).

Chemical Analysis

Platelet and membrane phospholipids were separated by thin layer chromatography (TLC) of $CHCl_3/CH_3OH$ extracts on Silica-Gel-HR-coated glass plates with a solvent system consisting of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25:15:4:2, v/v). The fatty acid compositions of the various fractions were determined by GC of the corresponding methyl esters prepared by transesterification with methanolic HCl. The results are reported here as the μmol of fatty acid/g protein, based on colorimetric protein analyses (12).

RESULTS AND DISCUSSION

Microviscosities of platelet membranes are presented graphically in Figure 1 as logarithmic functions of the reciprocal of the absolute temperature. Each straight line in Figure 1, plotted by least squares fit of the exponential function, represents 9-16 data points, which were omitted for clarity. Correlation coefficients for these lines appear in the figure legend. Dietary influences on membrane fluidity are evident, particularly at physiologically important temperatures. The values for η at 37 C of 3.5, 3.4, 2.8 and 2.2 poise for plasma membranes of platelets isolated from rabbits fed diets with cocoa butter, milkfat, coconut oil, or corn oil, respectively, reflect the relative viscosities of these fats.

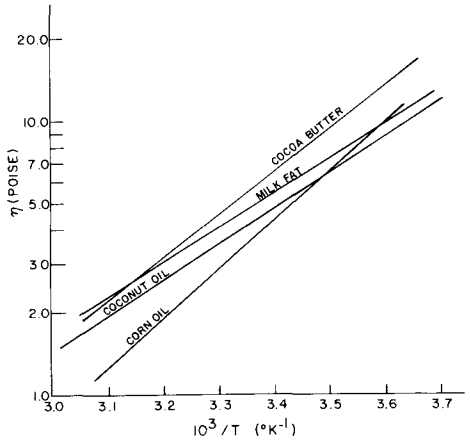

FIG. 1. Logarithmic plot of microviscosity vs the reciprocal of the absolute temperature for plasma membranes isolated from platelets from rabbits fed diets containing different fats. Correlation coefficients for these logarithmic functions are: cocoa butter, r= 0.9956; milkfat, r=0.9787; coconut oil, r=0.9732; and corn oil, r=0.9852.

The fatty acid compositions of the membrane phospholipids are given in Table II, both as the μmol fatty acid/g protein and as the mol %. The percentage distribution data reflect the fatty acid compositions of the ingested fats. The diet-induced differences in fluidity correspond to the relative percentages of saturated and polyunsaturated fatty acids in the phospholipids of these platelet membranes. Apparently the phospholipids of the platelet membranes of rabbits were lower with the cocoa butter or milkfat diet than with the coconut oil or corn oil diet. This difference suggests that the higher microviscosities of the membrane fractions from the rabbits fed cocoa butter and milkfat might result in part from the rigidity-inducing influences of the membrane proteins (9) in addition to the effects of fatty acid saturation per se. Comparison of the u/s ratios (Table II)

TABLE II

Fatty Acid Composition of Platelet Membrane Phospholipids

Diet	Cocoa butter		Milkfat		Coconut oil		Corn oil	
	μmol/ g protein	mol %	μmol/ g protein	mol %	μmol/ g protein	mol %	μmol/ g protein	mol %
Acid[a]								
14:0	–	–	–	–	–	–	11.68	1.53
16:0	108.22	29.92	103.02	28.10	129.25	23.94	174.22	22.81
16:1	–	–	0.68	0.19	4.82	0.89	12.84	1.68
18:0	108.13	29.89	109.59	29.89	146.70	27.18	201.32	26.36
18:1	48.08	13.29	53.22	14.52	42.91	7.95	53.67	7.03
18:2	48.96	13.54	50.56	13.79	108.14	20.03	168.30	22.04
20:0	2.04	0.56	1.26	0.34	2.99	0.55	2.59	0.34
20:4	31.72	8.77	27.58	7.52	65.49	12.13	73.42	9.61
22:0	3.47	0.96	2.90	0.79	8.11	1.50	8.51	1.11
24:0	0.85	0.23	2.78	0.76	8.25	1.53	15.80	2.07
24:1	10.24	2.83	15.06	4.11	23.17	4.29	41.36	5.42
u/s ratio[b]	0.62		0.68		0.83		0.84	

[a]No detectable amounts of the following fatty acids were present in any membrane phospholipid sample: 8:0, 10:0, 12:0, 15:0, 18:3, 20:3 and 22:6.

[b]u/s Ratio is calculated by dividing the total mol unsaturated fatty acid by the total mol saturated fatty acid.

and the microviscosities at 37 C for the membranes shows that unsaturation is important but is not the only determinant of membrane fluidity.

The fatty acid distributions in the various phospholipid classes are shown graphically in Figures 2-6. The amounts of material available for analysis were limited and complete distribution data were only obtained for plasma membrane phospholipids from milkfat- and cocoa-butter-fed rabbits and for whole platelet phospholipids from corn-oil- and coconut-oil-fed rabbits. Nevertheless, a comparison of these data for the phospholipid classes with the total phospholipid data (Table II) suggests that the phosphatidylcholine (PC) fraction is the most representative of the membranes. This is the largest fraction and its fatty acid distribution is most similar to that of the total phospholipids. The diet-induced differences in fatty acid unsaturation in the PC and phosphatidylethanolamine (PE) fractions alone are in consonance with the observed fluidities. Jakubowski and Ardlie (13) reported that only the PC and sphingomyelin fatty acids differed significantly in platelets from human subjects fed diets enriched with saturated or polyunsaturated fatty acids.

From a series of studies with rats, rabbits and man, Renaud and Gautheron (1,14) concluded that long chain saturated fatty acids, particularly stearic, and, to a lesser degree, palmitic, are thrombogenic. They reported (14) correlations between blood clotting and the ratio of stearic-to-linoleic acids in the dietary fats of laboratory rabbits. Renaud et al. (15) reported that these so-called "thrombogenic diets" affected the fatty acid composition of platelet phospholipids of rats such that a diet high in stearic acid led to a significant increase in the plasma and platelet phospholipids of the monounsaturated fatty acids, oleic and palmitoleic, with a corresponding decrease in the polyunsaturated fatty acids, linoleic and arachidonic. Our data (Table II) show higher percentages of oleic acid in the platelet membrane phospholipids from the rabbits fed cocoa butter or milkfat than from those fed coconut oil or corn oil, thus extending the findings with rats (15) to rabbits and allowing us to localize

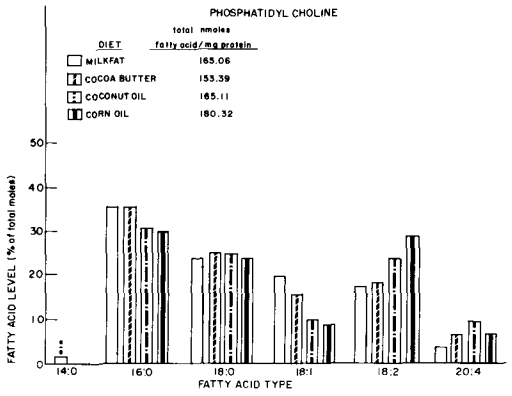

FIG. 2. Fatty acid composition of the phosphatidylcholine fraction from platelet plasma membranes of milkfat- and cocoa-butter-fed rabbits and whole platelets of corn-oil- and coconut-oil-fed rabbits.

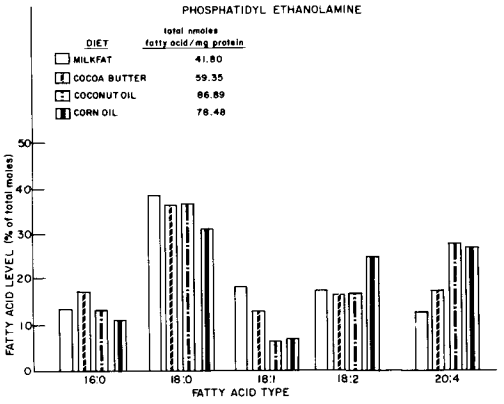

FIG. 3. Fatty acid composition of the phosphatidylethanolamine fraction of platelet plasma membranes of milkfat- and cocoa-butter-fed rabbits and whole platelets from corn-oil- and coconut-oil-fed rabbits.

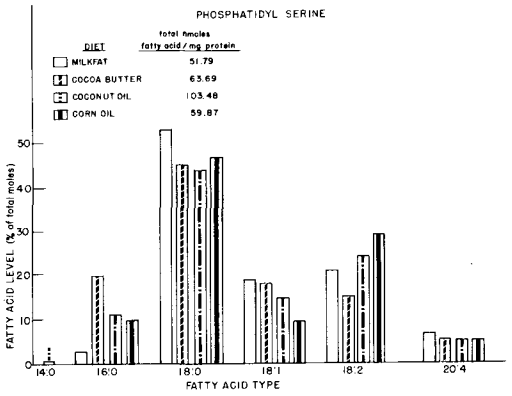

FIG. 4. Fatty acid composition of the phosphatidylserine from platelet membranes from milkfat- and cocoa-butter-fed rabbits and platelets from corn-oil- and coconut-oil-fed rabbits.

these effects on phospholipid fatty acids in the plasma membrane of the platelet.

In this study, we demonstrated nutritional modification of the fatty acid composition and physical properties of a mammalian cell membrane through dietary manipulations of the whole animal. Physical properties of membranes of altered compositions have been reported, usually with microorganisms or cultured cells (16,17). In contrast, this study and some published works (13,18) report on cell membrane modification in humans and animals through nutritional means. Extensive further work is necessary to determine the mechanisms that effect these changes and to determine what correlations exist between platelet membrane fluidity and platelet physiological properties that may ultimately relate to the potential for thrombosis.

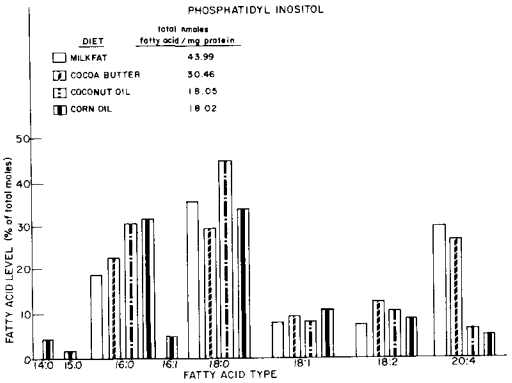

FIG. 5. Phosphatidylinositol fatty acid composition for platelet membranes from milkfat- and cocoa-butter-fed rabbits and platelets from corn-oil- and coconut-oil-fed rabbits.

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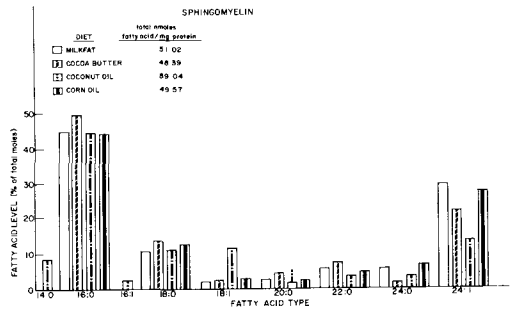

FIG. 6. Sphingomyelin fatty acid composition for platelet membranes from rabbits fed cocoa butter and milkfat and for platelets from rabbits fed corn oil and coconut oil.

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Biosynthesis of Hydrocarbons by Algae: Decarboxylation of Stearic Acid to N-Heptadecane in *Anacystis nidulans* Determined by ^{13}C- and ^{2}H-Labeling and ^{13}C Nuclear Magnetic Resonance[1]

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ABSTRACT

The distribution of isotopic labels in *n*-heptadecane enriched from [1,2-^{13}C] and [2-^{13}C, 2-$^{2}H_3$] acetates by *Anacystis nidulans* has been determined by ^{13}C nuclear magnetic resonance (^{13}C NMR). Labeling with [1,2-^{13}C] acetate is consistent with assembly from ^{13}C-^{13}C units derived from an acetate "starter" group and 8 malonate units, as in fatty acid biosynthesis, followed by production of a methyl group through bond cleavage of the terminal ^{13}C-^{13}C unit. A comparison of the hydrocarbon with palmitic acid (the only fatty acid produced in sufficient amount for NMR analysis) enriched from [2-^{13}C,2-$^{2}H_3$] acetate by the same culture shows that they have retained the same fraction of ^{2}H at corresponding sites, and have therefore undergone identical biosynthetic and hydrogen-deuterium exchange processes, as would be expected if *n*-heptadecane originates from de novo-synthesized stearic acid.

INTRODUCTION

The biosynthesis of hydrocarbons in microorganisms, plants and insects has received some attention but studies with algae are few (1-5). In bacteria, the formation of *n*-alkanes is believed to proceed by a modified condensation-reduction pathway involving head-to-head condensation between a fatty acid ester and the alkenyl residue of an α,β-alkenyl ether diacylglycerol (1), whereas in plants and insects it involves fatty acid elongation followed by decarboxylation (1). Investigations of the elongation-decarboxylation pathway in algae using radiotracers (4) have detected decarboxylases capable of converting fatty acid to *n*-alkane, but because of difficulties in determining the distribution of isotopic label they have not provided definitive evidence that these compounds have a common history, a necessary consequence if this is the main biosynthetic pathway. In this study, we have made a comparison between ^{13}C- and ^{2}H-enrichments detected by carbon-13 nuclear magnetic resonance (^{13}C NMR) in palmitic acid (reported in a recent paper [6]) and *n*-heptadecane, biosynthesized simultaneously by the blue-green alga *Anacystis nidulans* from [2-$^{13}C_{0.1}$,2-$^{2}H_{3.3}$] acetate (hereafter called [2-^{13}C,2-$^{2}H_3$] acetate. Labeled compounds are designated according to new IUPAC nomenclature (7). In reference 6, [2-$^{13}C_{0.1}$,2-$^{2}H_{3.3}$] acetate was called [2,2,2-$^{2}H_3$,2-$^{13}C_{0.1}$] acetate in accordance with provisional IUPAC rules). This, when considered with the labeling pattern observed on enriching the alkane with [1-$^{13}C_{0.1}$,2-$^{13}C_{1.0}$] acetate (hereafter called [1,2-^{13}C] acetate), establishes unequivocally that *n*-heptadecane originates by decarboxylation of de novo-synthesized stearic acid.

[1]NRCC No. 18251.

PROCEDURES

Materials

All solvents were glass-distilled before use. No grease, wax or "parafilm" was used. [2-^{13}C] and [1,2-^{13}C] Sodium acetate (both 90 atom %^{13}C) were obtained from Merck, Sharp and Dohme, Pointe Claire, Quebec. Sodium [2-^{13}C] acetate (90 atom % enriched) was exchanged 5 times with 4 mM $NaO^{2}H$ in $^{2}H_2O$ in a sealed stainless steel container at 110 C. Following this procedure, the [2-^{13}C,2-$^{2}H_3$] acetate always contained >98 atom %^{2}H by ^{13}C NMR analysis (see ^{13}C NMR section for details of NMR experiments).

Culture Conditions and Isolation Procedures for Metabolites

Wild-type *Anacystis nidulans* was maintained on agar slants 5 g/250 ml) prepared from Allen's medium (8). A production inoculum was prepared by transferring these cells to the Kratz and Myers liquid medium (9). After 5 days, the inoculum (200 ml) was added to the Kratz and Myers production medium (30 ℓ) in a special sterilized glass apparatus (10) and the cells were allowed to multiply at 41 C under constant illumination (16 fluorescent tubes;

100 W) and at pH 7.6. The isotopically labeled precursors were added to the culture (30 ℓ) 24 hr after inoculation and the cells allowed to multiply for a further 60 hr before harvesting. The cells were harvested by centrifugation, freeze-dried (yielding 12 g) and extracted with a mixture of ether/dichloromethane/methanol (5:3:2). The hydrocarbons were isolated from the crude extract (0.75 g) by chromatography first on silica gel and then on 20% silver nitrate-silica gel, with elution by hexane in each case. Gas liquid chromatography (GLC) showed the alkane mixure (0.22 g) was composed of *n*-pentadecane (17%), *n*-hexadecane (2.5%) and *n*-heptadecane (80.5%). *n*-Heptadecane was separated by preparative GLC as already described. The first silica gel column was stripped with methanol and the extract transesterified with potassium methoxide to yield a fatty acid ester fraction (0.15 g) which was purified by 20% silver nitrate-silica gel chromatography and shown by gas chromatographic analyses to consist of palmitic (94.0%), stearic (4.9%) and myristic acids (1.1%).

Gas Liquid Partition Chromatography

Gas chromatographic analyses were performed using a Hewlett Packard Model 5750 operating in the FID mode and equipped with glass columns (2 m x 4mm) packed with either 3% OV-1 on Gas Chrom Q or 10% SILAR-9CP on Chromosorb W. Using OV-1, operation was at 80 C for 8 min followed by programming at 4 C/min to 250 C; using SILAR-9CP, operation was programmed from 110 C to 250 C at 6 C/min. In both cases, the carrier gas was helium delivered at a flow rate of 30 ml/min; injection port and detector temperatures were set at 270 C and 300 C, respectively. Preparative GLC was carried out with a Hewlett Packard Model 5750 equipped with a 9:1 stainless steel splitter, operating in the FID mode, and using stainless steel columns (3.66 m x 6.4 mm) packed with 10% SILAR-9CP on Chromosorb W. Operation was the same as for analytical runs already described.

^{13}C NMR

^{13}C NMR spectra of *n*-heptadecane from *A. nidulans* were recorded at 25.16 MHz with a Varian XL-100/15 spectrometer equipped with a Varian 620L computer and Diablo disk accessory and with 5-mm diameter sample tubes and a sample temperature of 30 C. The ^{1}H decoupling frequency was 100 MHz and $\gamma H_2/2\pi$ was ca. 3500 Hz, with broad-band irradation by 0° to 180° phase modulation at 150 Hz. When used, ^{2}H decoupling was at 15.36 MHz, with $\gamma H_2/2\pi$ ca. 310 Hz and with phase modulation at 40 Hz. The spectral width (SW), acquisition time (AT), flip angle (FA) (90° pulse length 44 μsec), delay between acquisitions (PD) (during which the ^{1}H decoupler was switched off) and the time constant (TC) for weighting the free induction decay were the only parameters varied. Conditions for individual samples were: (a) *n*-heptadecane at natural ^{13}C-abundance, 15 mg in 0.25 ml C^2HCl_3 (Fig. 1a), ^{2}H internal lock, ^{1}H decoupling, SW 5120 Hz, AT 0.8s, FA 40°, PD 0s, TC -0.8s; (b) *n*-heptadecane labeled with [1,2-^{13}C]acetate, 17 mg in 0.25 ml C^2HCl_3 (Fig. 1b, yielding $^{1}J_{CC}$ in Table I), ^{2}H internal lock, ^{1}H decoupling, SW 2560 Hz, AT 3.2s, FA 40°, PDO, TC -1.6s; (c) *n*-heptadecane labeled with [1,2-^{13}C]acetate, 17mg in 0.25 ml 5:1 C^2HCl_3/C_6F_6, with 10 mg/ml chromium acetylacetonate (yielding intensity ratios in Table I), ^{19}F internal lock to C_6F_6, ^{1}H decoupling gated, SW 2560 Hz, AT 3.2s, FA 40°, PD 3.2s, TC -0.8s; (d) *n*-heptadecane labeled with [2-^{13}C,2-$^{2}H_3$]acetate, 22 mg in 0.4 ml 5:1 C^2HCl_3/C_6F_6 (Fig. 1d), ^{19}F internal lock, ^{1}H and ^{2}H decoupling, SW 1100 Hz, AT 14.9s, FA 54°, PD 0s, TC -10s, -6s, +10s (Fig. 1d not weighted), and in a separate experiment with ^{1}H decoupling only, AT 3.2s, FA 24°, TC -3.2s (to confirm which carbons are bonded to ^{2}H); (e) the sample just given with 10 mg/ml chromium acetylacetonate, ^{19}F internal lock, ^{1}H (gated) and ^{2}H decoupling, SW 1100 Hz, AT 3.2s, FA 54°, PD 6.8s (Fig. 1c), 26.8s, TC -10s, -1.6s (yielding $\%^{13}C$, $p_{^{13}C}$ and the proportions of isotopic species in Table I, and obtained with essentially the same experimental conditions reported in reference 6 to get the same type of data from the [^{13}C,^{2}H] labeled palmitic acid isolated from the same culture).

Enrichments and proportions of labeled species for *n*-heptadecane labeled with [2-^{13}C,2-$^{2}H_3$]acetate are averages measured from 3 separate spectra, digital integrations of each being carried out over expanded portions (25-200 Hz width) of plots obtained with 2 different values of TC (-10s and -1.6s). The absolute $\%^{13}C$ (Table I) was found by scaling the total integrated intensity I of each resonance to that of C-2, C-16, (superposed), both of which are at natural ^{13}C-abundance. The probability $p_{^{13}C}$ of ^{13}C from the 90% ^{13}C-enriched precursor is 0.9x, where x (the mole fraction of metabolite originating from the enriched precursor) is found from the equation $0.9x - 0.011(1-x) = \%^{13}C/100$. To obtain the proportions of species, the natural ^{13}C-abundance contribution 0.011(1-x) was subtracted from the proportion of ^{13}C not bonded to ^{2}H.

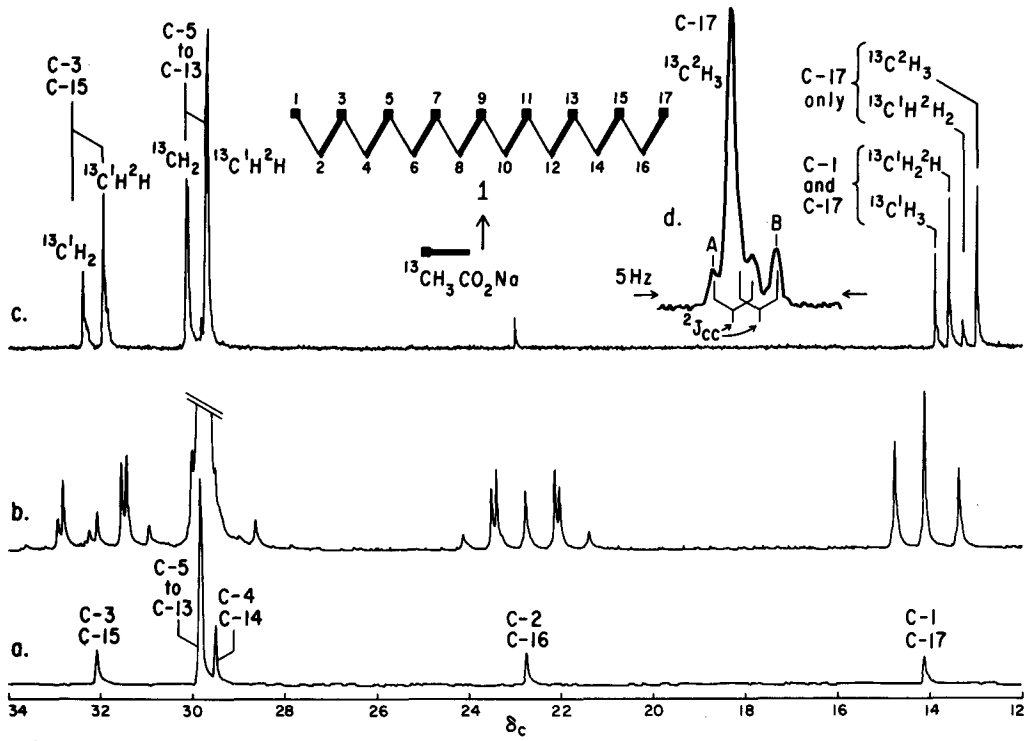


FIG. 1. ^{13}C Spectra of heptadecane (1) from *A. nidulans:* (a) at natural isotopic abundance; (b) from cultures supplemented with [1,2-^{13}C] acetate; (c and d) from cultures supplemented with [2-^{13}C,2-$^2H_3$] acetate; d is the expanded resonance of the $^{13}C^2H_3$ species at C-17. See Procedures for experimental details. Chemical shifts are altered slightly in c because of the addition of chromium acetylacetonate; d was recorded before this addition to resolve the satellite components A and B (see Results).

RESULTS

The ^{13}C resonances of *n*-heptadecane are readily assigned (Table I) from chemical shift calculations (11,12) or by comparison with those of the terminal carbons C-10 to C-18 of methyl stearate (13), allowing for slight downfield shifts resulting from C_6F_6 present in the solvent to provide the ^{19}F field-frequency lock.

In the ^{13}C spectrum (Fig. 1b) of *n*-heptadecane labeled with [1,2-^{13}C] acetate (composition 81% $^{13}CH_3{}^{13}CO_2^-$, 9% $^{13}CH_3{}^{12}CO_2^-$, 9% $^{12}CH_3{}^{13}CO_2^-$, 1% $^{12}CH_3{}^{12}CO_2^-$), the resonances for C-2 and C-16 consist of a singlet (integrated intensity I_s) caused by incorporation of singly ^{13}C-labeled units, 2 doublets arising from ^{13}C-^{13}C coupling between C-16 and C-17 (I_{d_1}) or C-2 and C-3 (I_{d_2}), and a quartet (I_q) caused by coupling among 3 adjacent ^{13}C nuclei at C-1, C-2, C-3 or C-15, C-16, C-17. Half of the quartet intensity overlaps the singlet to form the central peak. The ratio $I_q/(I_q + I_d)$ for the C-2 and C-16 resonances is 0.26 ± 0.02 (Table I), where $I_d = I_{d_1} + I_{d_2}$. This ratio equals the probability of incorporation of ^{13}C adjacent to an intact ^{13}C-^{13}C unit. Also, the proportion of ^{13}C bonded to one or more ^{13}C is given by $(I_d + I_q)/(I_s + I_d + I_q) = 0.92 \pm 0.01$, which is exactly that expected if 90% of the ^{13}C atoms at C-2, C-3 or C-16, C-17 were incorporated as ^{13}C-^{13}C units and the remaining 10% at C-2 or C-16 were single ^{13}C atoms adjacent to 24% ^{13}C (from the average $I_q/[I_q + I_d]$ for the C-2, C-16 and C-3, C-15 resonances, Table I) and 76% ^{12}C at C-1 or C-15: $I_{d_1} = I_{d_2} \propto (0.9 \times 0.76) + (0.1 \times 0.24)$; $I_q \propto 0.9 \times 0.24 \times 2$; $I_s \propto 0.1 \times 0.76 \times 2$; predicted $(I_d + I_q)/(I_s + I_d + I_q) = 0.924$.

Thus, [1,2-^{13}C] acetate (90% ^{13}C) is diluted 3.75-fold (0.9/0.24) with endogenous acetate before incorporation into *n*-heptadecane, scrambling of isotopic label is negligible and dilution of the enriched hydrocarbon by natural ^{13}C-abundance material is undetectable.

The average ^{13}C enrichment at C-1, C-2, C-16 and C-17 is 1.06 ± 0.04 times the average at C-3, C-15. This difference probably results from an acetate "starter" effect, as observed in [^{13}C,^2H] labeled methyl palmitate (6), where the enrichment at C-16, C-17 of the hydro-

TABLE I

^{13}C Chemical Shifts (δ_c), Isotope Chemical Shifts, ^{13}C Enrichments (% ^{13}C), Probabilities ($p_{^{13}C}$) of ^{13}C, $^{1}J_{cc}$ and Intensity Ratios for *n*-Heptadecane Labeled with ^{13}C and ^{2}H from Acetate

	Precursor [2-^{13}C,2-$^{2}H_3$]acetate						Precursor: [1,2-^{13}C]acetate[h]			
Carbon	δ_c[a] (ppm)	$\Delta\delta_c$[b] (ppm)	Total ^{13}C[c] (%)	$p_{^{13}C}$[d,e]	Proportions of species[f]		$^{1}J_{cc}$(Hz) for coupled pairs shown		$\frac{I_d + I_q}{I_s+I_d+I_q}$	$\frac{I_q}{I_d+I_q}$
C-17	14.37	0.30 0.60	22.7	0.219	$\frac{^{13}C^2H_3}{0.43}$	$\frac{^{13}C^1H^2H_2}{0.08}$	36.7	C-1, C-2;	0.586	–
C-1		0.90	±1.7	±0.017	$\frac{^{13}C^1H_2{}^2H}{0.34}$	$\frac{^{13}C^1H_3}{0.15}$	±0.3	C-16, C-17	±0.007	
C-16 C-2	23.45						34.5 ±0.3	C-2, C-3; C-15, C-16	0.92 ±0.01	0.26 ±0.04
C-15 C-3	32.79	0.43	20.0 ±1.7	0.191 ±0.017	$\frac{^{13}C^1H^2H}{0.31_5}$	$\frac{^{13}C^1H_2}{0.68_5}$	34.7 ±0.3	C-3, C-4; C-14, C-15	0.92 ±0.01	0.22 ±0.04
C-14 C-4	30.22						g		g	g
		Averages for odd numbered carbons								
C-13 to C-5	30.56 to 30.52	0.38	21.1 ±1.0	0.202 ±0.010	0.30	0.70	g		g	g

[a] ^{13}C Chemical shifts referred to internal $(CH_3)_4Si$, solvent C^2HCl_3/C_6F_6 = 6/1, no chromium acetylacetonate added. Shifts δ_c of carbons not shown in table are: C-12 to C-6 (even numbers) 30.52, 30.56, error ±0.01 ppm.

[b] ^{2}H-Induced isotope shift (upfield), error ±0.02 ppm.

[c] Including natural-abundance material. Chromium acetylacetonate added to solution. Average of 3 spectra. Error shown is for comparison of 2-% ^{13}C values; absolute error is ca. 2.0% higher because of error in integrals of weak natural-abundance peaks (C-2, C-16).

[d] Probability of ^{13}C from precursor (an average for equivalent carbons), after subtracting contribution from natural abundance material. Average of 3 spectra.

[e] Comparative error shown; for absolute error add ca. ±0.020.

[f] Average for equivalent carbons. Error <±0.02 except C-3, C-15 ± 0.01_5. Contribution from natural-abundance material subtracted.

[g] Not measurable because peaks overlap. The average integrated intensity for these resonances equals the average for C-2, C-16 and C-3, C-15. Hence, the average ^{13}C probability (P) for C-4 through C-14 = $I_q/(I_d + I_q)$ for C-2, C-16, C-3, C-15 = 0.24 ± 0.04.

[h] Chromium acetylacetonate added to solution for measurements of intensity I; s = singlet, d = doublet, q = quartet.

carbon is 1.12 ± 0.08 times higher than at C-1, C-2. Since no scrambling of the precursor is detectable, the average $I_d/(I_s + I_d)$ (Table I, $I_q = 0$) for C-1 and C-17 (0.586 ± 0.007) indicates that an intact doubly labeled unit is incorporated at one end (C-16, C-17) only. Hence, $I_d/(I_s + I_d)$ is 0.9 for the C-17 resonance, and for the C-1 resonance it is (2.12 x 0.586) - (1.12 x 0.9) = 0.23 ± 0.03. The 0.23 ± 0.03 value equals, within error, the probability of adjacent incorporation of ^{13}C-^{13}C units at C-2, C-3; C-4, C-5; C-14, C-15 and C-16, C-17, just determined from $I_q/(I_d + I_q)$ for the resonances of C-2, C-16 and C-3, C-15 (average 0.24 ± 0.04, Table I). Thus, the (^{13}C-^{13}C)-labeled precursor pool giving rise to pairs of methylene carbons such as C-2, C-3 and C-14, C-15 also provides the ^{13}C-^{13}C unit from which C-1 is produced by bond cleavage and loss of a carbon. Although it is not possible to show directly that C-6 through C-13 were incorporated as ^{13}C-^{13}C units (spin-spin couplings are not measurable between nuclei with the same chemical shift), the average ^{13}C probability (P) for these carbons (0.24 ± 0.04, Table I, footnote g) is in full accord with their having been derived from the same precursor pool. Uniform labeling of C-1 through C-15 is consistent with successive addition of 8 2-carbon units from a common precursor pool (with P = 0.24 ± 0.04) to the C-16, C-17 "starter" group, followed by cleavage of the terminal carbon-carbon single bond to give C-1. This would be expected if *n*-heptadecane were obtained from de novo-synthesized stearic acid, and further evidence is provided by the labeling experiment with [2-^{13}C,2-$^{2}H_3$]acetate.

Cultivation of *A. nidulans* with exogeneous [2-^{13}C,2-$^{2}H_3$]acetate yielded hydrocarbons and fatty acids multiply labeled with ^{13}C and ^{2}H. The isotopic distribution in palmitic acid isolated from this experiment has been reported previously (6) and is now compared with results obtained from the analysis of the ^{1}H,^{2}H-broad-band decoupled ^{13}C spectrum (Fig. 1c) of labeled *n*-heptadecane (Table I). Insufficient stearic acid was obtained for NMR analysis. Although it might at first appear more appropriate to match its isotopic distribution with that of *n*-heptadecane, no more information would have been obtained because the isotopic distribution at C-4 of stearic (or palmitic) acid is not measurable because of peak overlap (6). Thus, C-1,C-15 and C-17 of *n*-heptadecane could be compared with C-2, C-16 and C-18 of stearic acid, or C-3, C-15 and C-17 with C-2,C-14 and C-16 of palmitic acid. The comparison C-3, C-15 and C-17 with C-2,C-14 and C-16 of palmitic acid is valid because we have shown that C-1 of *n*-heptadecane was introduced by the same processes as C-2 through C-15, a conclusion which receives further proof from the results to follow. Odd-numbered positions only were enriched with both ^{13}C and ^{2}H in *n*-heptadecane and the presence of one isotopically-shifted component for each of the resonances of odd-numbered carbons from C-3 to C-15 established that these carbons bore not more than one ^{2}H atom. Moreover, the fraction of the theoretical maximum ^{2}H retention $f_{2H} = I(^{13}C^{1}H^{2}H)/(I[^{13}C^{1}H^{2}H] + I[^{13}C^{1}H_2])$ where I is the ^{13}C resonance intensity for the species shown, was 0.70 ± 0.02 when averaged for odd-numbered carbons from C-3 to C-15, in close agreement with the average f_{2H} for C-2 to C-14 of [^{13}C, ^{2}H]-enriched palmitic acid (0.68 ± 0.02) (6).

The resonance for C-1 and C-17 in [^{13}C, ^{2}H]-labeled *n*-heptadecane had 3 isotopically shifted components corresponding to the species $^{13}C^{2}H_3$, $^{13}C^{1}H^{2}H_2$ and $^{13}C^{1}H_2{}^{2}H$ in addition to the $^{13}C^{1}H_3$ signal (Table I). As the [1,2-^{13}C]acetate experiments showed that C-16, C-17 are incorporated as an intact ^{13}C-^{13}C unit and precluded C-1 from retaining the 3 methyl hydrogens of acetate, it follows that C-16, C-17 must originate from an intact acetate "starter" unit with C-17 giving rise to the $^{13}C^{2}H_3$ signal. Moreover, as $I(^{13}C^{2}H_3)$: $I(^{13}C^{1}H^{2}H_2) = 5.4$ (Table I) is the same as the corresponding ratio of 5.6 found for the methyl group of [^{13}C,^{2}H]-labeled palmitic acid from the same culture (6), it is probable that the distribution of isotopic species at C-16 of palmitic acid (or C-18 of stearic acid), namely 0.79:0.14:0.04: ⩽ 0.03 = 0.43:0.08:0.02: ⩽0.02, is the same as at C-17 of *n*-heptadecane. The observed isotopic distribution for C-1 plus C-17 (Table I) minus the contribution just stated for C-17 gives $I(^{13}C^{1}H_2{}^{2}H)$:$I(^{13}C^{1}H_3)$ for C-1 of labeled *n*-heptadecane, bearing in mind that the $^{13}C^{2}H_3$ and $^{13}C^{1}H^{2}H_2$ signals have no contribution from C-1. Thus, $I(^{13}C^{1}H_2H)$:$I(^{13}C^{1}H_3)$ for C-1 = (0.43:0.08:0.34:0.15) - (0.43:0.08:0.02:⩽0.02) = 0.32:0.13 = 0.71: 0.29 (± 0.05), in agreement with the corresponding ratio of 0.73:0.27 (± 0.02) obtained for C-2 of palmitic acid (6). Also, as the intensities of C-1 and C-17 are separable, it follows that the acetate "starter" effect is $p_{^{13}C}$(C-17):$p_{^{13}C}$(C-1) = (0.43 + 0.08 + 0.02 + 0.02)/(0.32 + 0.13) = 1.22 ± 0.15, which is close to that ($p_{^{13}C}$[C-16]: $p_{^{13}C}$[C-2] = 0.138/0.112 = 1.23 ± 0.11) found for palmitic acid (6).

The resonance component for $^{13}C^{2}H_3$ at C-17 of *n*-heptadecane showed satellites resulting from 2-bond ^{13}C-^{13}C coupling with C-15

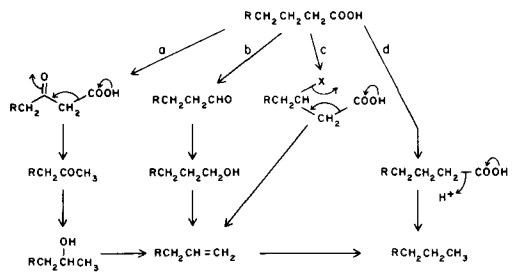
($^2J_{CC}$ = 1.0 ± 0.1 Hz, Fig. 1d). As a portion of these satellites was isotopically shifted by ^2H at C-15, their intensity relative to the unshifted satellites directly measures f_{2H} at C-15 without interference from the C-1 resonance which has no $^{13}C^2H_3$ component. One branch of each of these satellites (denoted A and B in Fig. 1d) was clearly distinguishable in a spectrum obtained with high resolution (AT 14.9s, no relaxation reagent added) and good signal/noise. Several measurements of both integrals (I) and peak heights (H) from highly-expanded plots (5 Hz corresponding to 50 cm) using different values of TC (see Procedures), yielded averages for $I_B/(I_A + I_B)$ = 0.637 ± 0.021 SD and $H_B/(H_A + H_B)$ = 0.624 ± 0.017 SD. The overall average (0.630 ± 0.019 SD) was taken for f_{2H} at C-15, in view of the good agreement between the 2 sets of measurements. Small differences in spin-lattice relaxation time between the 2 sets of satellites might occur because of the presence, in one case, of ^2H 3-bonds removed (although this is unlikely since relaxation would be dominated by the 2 protons 2 bonds removed). Nevertheless, under the conditions used (long AT), any effect on satellite intensities would be negligible. Differences resulting from second order spin-spin coupling effects should also be insignificant, since $^1J_{CC}$(1.0Hz) $<<$25.16 (δ_C[C-1, C-17]-δ_C [C-3, C-15]). Similar satellites occurred about the $^{13}C^1H^2H_2$ component of the C-17 resonance (Fig. 1) but the lower intensity precluded accurate measurements. This value of f_{2H} for C-15 (0.630 ± 0.019) agrees with that for C-14 of labeled palmitic acid (0.63 ± 0.02); similarly f_{2H} for C-3 (0.74 ± 0.05, since the average f_{2H} for C-3 and C-15 is 0.685 ± 0.015) compares well with f_{2H} for C-2 of palmitic acid (0.73 ± 0.02).

The probability p_A% of adjacent incorporation of ^{13}C-labeled units is calculable from the relative intensity of 3-bond isotopically-shifted components of the C-17 and C-15, C-3 resonances, where they were well-resolved. The average p_A (39 ± 5%) is larger than the average p_{13C} (ca. 20%), showing that the [^{13}C,^2H]-labeled hydrocarbon sample was diluted by natural ^{13}C,^2H-abundance material. The corresponding values for palmitic acid (6) (p_A 58 ± 10%, p_{13C} ca. 13%) indicate a higher incorporation and a greater degree of natural ^{13}C, ^2H-abundance dilution.

DISCUSSION

Although previous studies with blue-green algae (4) suggested that medium length *n*-alkanes (e.g., C_{15}, C_{17}) originate from open-chain fatty acids by decarboxylation, definitive evidence from radiotracer experiments was lacking because of the difficulty of pinpointing the incorporated label in these symmetrical molecules. The introduction of cryptic asymmetry by labeling with [1,2-^{13}C]-and [2-^{13}C, 2-$^2H_3$]acetate, however, permits enrichment information from both ends of the molecule to be obtained by ^{13}C NMR techniques. The results of the [1,2-^{13}C]acetate experiment were analogous to those expected for fatty acid biosynthesis, and are clearly consistent with the formation of *n*-heptadecane from a starter acetate group C-16, C-17) and 8 malonate units, with bond cleavage of the terminal ^{13}C-^{13}C unit to give C-1. The fatty acid origin of *n*-heptadecane is also suggested by the ^{13}C-enrichment of the odd-numbered carbons by [2-^{13}C,2-$^2H_3$]acetate; these carbons bore *at most one deuterium, except* C-17 which was bonded to 3. Most importantly, the *same* fraction of deuterium is retained at corresponding sites in palmitic acid produced concurrently by *A. nidulans*, requiring all biosynthetic and hydrogen-deuterium exchange processes to be identical for both types of metabolites. This proves that *n*-heptadecane is obtained from de novo-synthesized stearic acid and that palmitic and stearic acids are produced by the same biosynthetic process in *A. nidulans*.

The mechanism and stereochemistry of the decarboxylation process is as yet unresolved. Several mechanisms have been suggested (1-3) and these are summarized in Scheme I:



SCHEME I

Three of these routes (a-c) invoke the generation of an intermediate vinyl group which may subsequently be reduced to the alkane. Two hydrocarbons, each containing a vinyl group, have been reported from *Chlorella pyrenoidosa* (3) and *Botryococcus braunii* (1). We did not detect the presence of a similar alkene in *A. nidulans*, although our results rule out oxidative decarboxylation processes a and b since both of these would be likely to decrease the deuterium content of C-2 in the fatty acid

(C-1 in the hydrocarbon) in complete contrast with the experimental findings. On the other hand, route c, which promotes decarboxylation with a leaving group (e.g., OH) at the β position, would not necessarily disturb the deuterium enrichment. Route d is simply a direct decarboxylation process that requires no intermediate.

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Characteristics of Phospholipids in Human Lung Carcinoma

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ABSTRACT

Human lung carcinoma tissues with histological types of adenocarcinoma, squamous cell and small cell carcinoma were investigated for phospholipids. There were marked differences in the phospholipids between these lung carcinoma and normal lung tissue. A marked decrease in saturated phosphatidylcholine (PC), predominantly the dipalmitoyl species, was noted in the carcinoma, although they still contained 17-20% of the saturated classes. The lung carcinoma contained less phosphatidylglycerol (PG) and lyso-*bis*-phosphatidic acid and more cardiolipin and phosphatidylinositol (PI) than the normal lung tissue. These alterations observed in the lung carcinoma appeared to show that they lose the characteristic feature of phospholipids in the lung tissue. The differences in the lipid composition among different cell types of lung carcinoma were also noted. The squamous cell and small cell carcinoma contained more triacylglycerol and relatively higher dienes I (monoenoic-monoenoic) and lower dienes II (saturated-dienoic) of PG, respectively, as compared to adenocarcinoma.

INTRODUCTION

Lung carcinoma is characterized by the occurrence of histologically various types reflecting the variety of their mother cells in the lung (1). In order to elucidate the nature of different kinds of carcinoma, it is important to obtain various information from different fields of research. A number of studies on the lipid analysis or lipid metabolism of carcinoma tissues have been made to assess the biochemical nature of the membranes in carcinoma (2-7). These lipid-biochemical studies on the carcinoma tissues have been carried out mainly in hepatoma (5-7). However, there has been no report on the lipid analysis of human lung carcinoma. Therefore, this study aims to elucidate the characteristics of phospholipids, particularly the molecular structure of phosphatidylcholine (PC), in lung carcinoma of histologically different types. This is the first description of the phospholipids of human lung carcinoma.

MATERIALS AND METHODS

Materials

Lung carcinoma tissue and normal lung tissue were obtained from separate patients operated on in the Sapporo Medical College Hospital in Sapporo, Japan. The normal lung tissue samples were taken from the non-tumorous portions in lungs excised by pneumonectomy or lobectomy. Histological examination indicated that these lung tissues were normal. The lung carcinoma tissues were histologically divided into 3 groups: squamous cell carcinoma, small cell carcinoma and adenocarcinoma. It was confirmed by histological examination that none of the adenocarcinoma were derived from type II cells. The patients with these lung carcinoma had had no special chemotherapy or radiation before the operation which might have conceivable altered lipid composition. The lung carcinoma were carefully freed from surrounding as well as necrotic and hemorrhagic areas.

Lipid Extraction and Fractionation of Major Lipid Classes

The lung carcinoma thus obtained and the normal lung tissues were immediately homogenized in 0.25 M sucrose in 0.01 M Tris-HCl buffer (pH 7.4) and an aliquot was taken for determination of protein. The lipids were extracted from the homogenates by the Bligh and Dyer method (8). The total lipids were divided into 3 major lipid classes by chromatography on a DEAE-cellulose column as described elsewhere (7,9). The stepwise elution from the column was carried out as follows: neutral lipids with chloroform, neutral phospholipids with methanol and acidic phospholipids with chloroform/methanol (2:1) saturated with ammonia hydroxide, respectively. More than 90% of the lipid phosphorus applied to the column was recovered by this procedure. Neutral phospholipids were fractionated by thin layer chromatography (TLC) with chloroform/methanol/water (70:30:5, v/v). Five spots were detected under ultraviolet (UV) light after spraying with 0.2% dichlorofluorescein in ethanol and were identified with authentic standards as phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin I (containing lignoceric acid as the main fatty acid), sphingomyelin II (containing stearic acid

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as the main fatty acid) and lysophosphatidylcholine in that order from the top of the plate. Acidic phospholipids were separated by 2-dimensional TLC as described by Rouser et al. (10). Identification of the phospholipids on the plates was made on the basis of ninhydrin (11) and Dittmer's (12) reagent spray, and by comparisons with the migration of reference standards. After chromatography, the spots on the plates were detected by charring with formalin-sulfuric acid reagent (13), and the lipid phosphorus of the spots were then determined.

Analysis of Phosphatidylcholine

PC was isolated from the neutral phospholipid fraction as already mentioned. The spot on the plate was scraped and eluted with chloroform/methanol/acetic acid/water (50:39:1:10, v/v) as described by Arvidson (14) and the extract was washed once with 50% methanol. The recovery of PC by this procedure was ca. 94% when examined in a separate experiment using radioactive PC.

PC was treated with phospholipase A_2 and the degradation products were isolated by TLC (15). Fatty acids and lysophosphatidylcholine were eluted from the gel with chloroform/methanol (2:98) for analysis of fatty acid composition.

Purified PC was hydrolyzed by phospholipase C from *Clostridium welchii*, as described by Renkonen (16). The 1,2-diacylglycerol formed was acetylated with acetic anhydride and anhydrous pyridine (17). The diacylglycerol acetates were purified and recovered as described by Kuksis et al. (18). The diacylglycerol acetates thus obtained were resolved into molecular species according to the degree of unsaturation by means of argentation TLC ($AgNO_3$-TLC) as described previously (9). By this 2-step development, the diacylglycerol acetates from the PC were separated into 7 subfractions designated as saturates, monoenes, dienes I (monoenoic-monoenoic), dienes II (saturated-dienoic), trienes, tetraenes and polyenes, respectively. Identification of the molecular species was achieved by cochromatography of standard materials or by the determination of fatty acid composition. The relative proportion of diacylglycerol acetates separated by $AgNO_3$-TLC was estimated by glycerol determination of each subfraction.

Subfractionation of 1,2-diacylglycerol by $AgNO_3$-TLC (19) was also performed to isolate monoenoic diacylglycerol, which was immediately converted to phosphatidylphenol (20). Phospholipase A hydrolysis of the phosphatidylphenol was done as described by Åkesson (20).

The proportion of diacyl, alkenyl-acyl and alkyl-acyl types in PC was determined by the combination of mild alkaline and acid hydrolysis methods as described previously (7).

Other Analytical Procedures

The fatty acid methyl esters of each lipid were prepared with BF_3/methanol (21). The fatty acid methyl esters were analyzed on a 2 m x 4 mm od pyrex column packed with 10% diethyleneglycol succinate polyester on Chromosorb W at 185 C. Identification of fatty acid methyl esters was made by comparison with the retention times of reference standards. In some cases, the diacylglycerol acetates were analyzed on a 50 cm x 4 mm od pyrex column packed with 1% Silicone OV-1 on Gaschrom Q at 290 C. The peak identity of the diacylglycerol acetates was determined by comparison with calibration standards composed of dimyristoyl, dipalmitoyl, distearoyl and diarachidoyl acetates. All gas chromatographies were done with a Shimadzu GC-5A gas chromatograph, equipped with a flame ionization detector, in conjunction with Shimadzu Chromatopac E1A.

Protein was determined by the Lowry et al. method (22) as the standard of bovine serum albumin. Lipid phosphorus was determined by the Bartlett method (23), glycerol by the Van Handel and Zilversmit method (24), free and esterified cholesterol by the Zak et al. (25) and free fatty acid by the Itaya and Ui method (26).

Student's t-test was used for statistical analysis of results. *P* values equal to or less than 0.05 were considered significant.

RESULTS

The content of major lipid constituents in the lung carcinoma is shown in Table I. Since differences in protein content/wet tissue were small for the normal and lung carcinoma tissues, the data are given on the basis of protein content. The amount of triacylglycerol in the lung carcinoma was highly variable, being ca. 2.5 times higher in squamous cell carcinoma and small cell carcinoma than in adenocarcinoma and the normal lung tissues. The amount of free fatty acid and phospholipid was almost similar in both the normal lung tissue and all types of the lung carcinoma, although the phospholipid of small cell carcinoma was somewhat higher ($P < 0.05$) than normal lung tissue. It has been reported that there was less phospholipid in the hepatoma from humans (27) and animals (4,7) than in control liver tissues. However, the phospholipid content in

the human lung carcinoma was almost the same as that in the lung tissue.

It was noted, however, that the composition of phospholipid was much different from that of the control lung tissue, particularly in the acidic phospholipids. Table II shows the composition of phospholipids from the normal lung tissue and from the lung carcinoma. No significant differences were observed in the neutral phospholipid composition of any of the types of lung carcinoma nor in normal lung tissue. The main phospholipids were PC and phosphatidylethanolamine (PE), and the content among them was also similar. However, marked differences existed in the acidic phospholipid composition between the normal lung tissue and the carcinoma. The identification of acidic phospholipids on the plates was made by comparison of reference standards and ninhydrin and Dittmer's reagent spray. However, lyso-*bis*-phosphatidic acid was tentatively identified by Dittmer positive, with its R_f value being comparable to the data described by Rouser et al. (28). An unidentified phospholipid was found in near origin. Significant decreases of phosphatidylglycerol and lyso-*bis*-phosphatidic acid and increases of phosphatidylinositol (PI) and cardiolipin were found in the lung carcinoma but not in normal lung tissues. However, no quantitative differences were observed among the cell types of the lung carcinoma, i.e., these changes in acidic phospholipids appear to be characteristic of the lipid composition of lung carcinoma.

The PC purified from human lung carcinoma as well as that from normal lung tissue was only of the diacyl type (data not shown). Although it has been reported that the alkyl type of PC occurs in elevated levels in many tumors (29,30), the alkyl or alkenyl types of PC apparently was not in human lung carcinoma.

The results of the fatty acid analyses of PC in the lung carcinoma and the normal lung tissue are presented in Table III. The fatty acids in the lung carcinoma were found to be qualitatively the same as those in the normal lung tissue. However, more significant decreases of 16:0 in the 2-position were observed in all types of lung carcinoma than in the normal lung tissue; 16:0 in the 1-position also decreased in squamous cell and small cell carcinoma. In contrast, increased 18:1 in the 2-position was found in all types of lung carcinoma, and 18:1 in the 1-position also increased in squamous cell and small cell carcinoma. That is, there was a reversed relationship between squamous cell and small cell carcinoma, and adenocarcinoma in the content of 16:0 and 18:1 in PC. It has been reported that, in gen-

TABLE I

Lipid Content of Human Lung Carcinoma of Histologically Different Types

	Lipid content (μg/mg protein)							
	Normal lung (n = 3)	Squamous cell carcinoma (n = 4)	t-test	Small cell carcinoma (n = 3)	t-test	Adenocarcinoma (n = 4)	t-test
Triacylglycerol	11.1 ± 2.2 (8.1)	34.5 ± 10.2 (22.3)	$P < 0.05$	33.1 ± 4.1 (17.7)	$P < 0.001$	14.1 ± 4.2 (8.2)	NS
Free cholesterol	30.5 ± 14.0 (22.3)	25.5 ± 7.5 (16.5)	NS	40.0 ± 11.0 (21.4)	NS	46.5 ± 12.0 (26.9)	NS
Esterified cholesterol	6.5 ± 1.5 (4.7)	7.5 ± 2.5 (4.8)	NS	10.0 ± 3.0 (5.4)	NS	9.5 ± 4.0 (5.5)	NS
Free fatty acid	0.35 ± 0.1 (0.3)	0.41 ± 0.1 (0.3)	NS	0.45 ± 0.1 (0.2)	NS	0.52 ± 0.1 (0.3)	NS
Phospholipid	88.4 ± 7.7 (64.6)	87.0 ± 9.8 (56.2)	NS	103.1 ± 4.1 (55.2)	$P < 0.05$	102.1 ± 9.8 (59.1)	NS

Values are means ± SD (n). Values in parentheses are percentage distribution among classes. NS: not significant.

TABLE II

Phospholipid Composition of Human Lung Carcinoma of Histologically Different Types

	Phospholipid composition (mol %)						
	Normal lung (n = 3)	Squamous cell carcinoma (n = 4)	t-test	Small cell carcinoma (n = 3)	t-test	Adenocarcinoma (n = 4)	t-test
Phosphatidylethanolamine	19.1 ± 1.4	21.1 ± 2.5	NS	21.0 ± 1.1	NS	20.9 ± 1.9	NS
Phosphatidylcholine	50.7 ± 1.0	54.3 ± 1.9	NS	50.8 ± 2.3	NS	50.1 ± 3.5	NS
Sphingomyelin-I	7.6 ± 1.7	6.0 ± 1.3	NS	6.4 ± 1.4	NS	6.3 ± 0.4	NS
Sphingomyelin-II	9.8 ± 1.6	5.8 ± 0.5	$P < 0.01$	7.1 ± 1.0	NS	8.3 ± 1.8	NS
Lysophosphatidylcholine	1.9 ± 0.6	1.2 ± 0.2	NS	4.0 ± 1.2	$P < 0.05$	3.0 ± 1.2	NS
Phosphatidylserine	0.5 ± 0.3	0.8 ± 0.6	NS	0.5 ± 0.4	NS	0.7 ± 0.4	NS
Phosphatidic acid	0.4 ± 0.2	0.3 ± 0.1	NS	0.6 ± 0.2	NS	0.5 ± 0.3	NS
Phosphatidylinositol	2.9 ± 0.2	5.1 ± 0.6	$P < 0.01$	5.0 ± 0.4	$P < 0.01$	4.0 ± 0.4	$P < 0.05$
Phosphatidylglycerol	2.7 ± 0.7	0.9 ± 0.1	$P < 0.01$	0.8 ± 0.1	$P < 0.05$	1.1 ± 0.5	$P < 0.05$
Cardiolipin	1.5 ± 0.1	2.8 ± 0.6	$P < 0.05$	2.7 ± 0.2	$P < 0.01$	3.6 ± 1.1	$P < 0.05$
Lyso-*bis*-phosphatidic acid	2.4 ± 0.2	1.0 ± 0.1	$P < 0.001$	0.8 ± 0.5	$P < 0.01$	0.9 ± 0.3	$P < 0.001$
Unidentified	0.4 0.2	0.7 ± 0.1	NS	0.3 ± 0.2	NS	0.7 ± 0.4	NS

Values are means ± SD (n). Sphingomyelin-I and -II contained lignoceric and stearic acids as a predominant fatty acid, respectively. NS: not significant.

TABLE III

Fatty Acid Composition of Phosphatidylcholine of Human Lung Carcinoma of Histologically Different Types

	Fatty acid composition (mol %)							
	Normal lung (n = 3)		Squamous cell carcinoma (n = 4)		Small cell carcinoma (n = 3)		Adenocarcinoma (n = 4)	
	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position
14:0	2.0 ± 0.3	2.2 ± 0.4	2.3 ± 0.2	1.7 ± 0.2	1.8 ± 0.3	1.0 ± 0.1	1.5 ± 0.6	1.1 ± 0.2
16:0	66.9 ± 3.9	32.1 ± 0.9	51.8 ± 6.5	22.8 ± 3.3	53.7 ± 5.4	19.0 ± 8.0	65.3 ± 4.7	20.9 ± 0.5
16:1	1.0 ± 0.2	6.6 ± 0.3	–	5.4 ± 0.5	0.9 ± 0.1	7.3 ± 1.5	0.6 ± 0.2	5.1 ± 2.1
18:0	14.6 ± 0.6	0.6 ± 0.2	19.6 ± 0.8	–	15.0 ± 6.2	2.2 ± 0.2	17.9 ± 2.9	–
18:1	11.5 ± 0.5	22.6 ± 0.5	22.4 ± 4.2	36.0 ± 9.5	25.5 ± 2.5	40.0 ± 5.9	11.0 ± 1.8	33.8 ± 7.5
18:2	3.8 ± 0.9	18.4 ± 0.2	3.9 ± 1.4	13.9 ± 0.9	3.1 ± 0.8	15.4 ± 6.0	1.9 ± 0.3	18.3 ± 9.1
20:1	–	–	–	–	–	1.0 ± 0.2	–	0.3 ± 0.1
20:3	–	1.9 ± 0.3	–	1.0 ± 0.1	–	2.3 ± 0.1	–	3.0 ± 0.3
20:4	–	10.4 ± 0.5	–	14.0 ± 3.6	–	7.2 ± 0.9	–	13.4 ± 3.1
20:5	–	2.2 ± 0.1	–	1.4 ± 0.2	–	3.2 ± 0.9	–	2.1 ± 0.8
22:6	–	2.5 ± 0.1	–	–	–	–	–	–

Values are means ± SD (n).

eral, hepatoma lipids contain higher levels of 18:1 and lower levels of 18:2 and polyunsaturated fatty acids (4). The human lung carcinoma studied also contained higher levels of 18:1, particularly in the 2-position, but the contents of 18:1 and polyunsaturated fatty acids were almost the same as those of the normal lung tissue. Furthermore, the marked changes in 16:0 content were observed in lung carcinoma as a characteristic feature.

Table IV gives the proportion of molecular classes, characterized by their degree of unsaturation, of PC from the lung carcinoma. In reflecting on the fatty acid profiles, the most striking characteristic in the molecular classes of PC was the marked decrease in saturated classes, the content of which was ca. 17-20% in all types of lung carcinoma. As shown in Table V, the fatty acid composition of the saturated PC isolated by $AgNO_3$-TLC was the same in normal lung and lung carcinoma. These PC are therefore quantitatively similar and predominantly dipalmitoyl in type. The content of monoenes was similar in both the normal lung tissue and lung carcinoma. The less selective distribution of fatty acids in the glycerol moiety in the tumor cells has been observed by several investigators (5,31). However, the disturbance of the positional specificity of fatty acids seemed to be insignificant in the monoenes of PC in all types of lung carcinoma (Table VI). These results indicate that a predominant molecular species of the monoenes is 16:0 at the 1-position and 18:1 at the 2-position, and that the amount of molecular species having 18:1 at the 1-position and 16:0 at the 2-position is relatively low and almost the same in all types of lung carcinoma. The content of dienes I and II were also similar in both the normal lung tissue and lung carcinoma. However, the squamous cell and small cell carcinoma contained somewhat higher dienes I (monoenoic-monoenoic) and lower dienes II (saturated-dienoic) of PC as compared to adenocarcinoma.

TABLE IV

Molecular Class Composition of Phosphatidylcholine of Human Lung Carcinoma of Histrologically Different Types

	Molecular class composition (mol %)							
	Normal lung (n = 3)	Squamous cell carcinoma (n = 4)	t-test	Small cell carcinoma (n = 3)	t-test	Adenocarcinoma (n = 4)	t-test
Saturates	31.4 ± 1.2	17.3 ± 2.2	$P < 0.001$	16.9 ± 4.1	$P < 0.01$	20.4 ± 3.0	$P < 0.01$
Monoenes	29.4 ± 1.6	34.3 ± 4.0	NS	31.0 ± 1.7	NS	32.2 ± 1.5	NS
Dienes-I (MM)	4.3 ± 1.2	8.9 ± 3.2	NS	12.9 ± 6.5	NS	4.1 ± 2.1	NS
Dienes-II (SD)	16.9 ± 2.0	12.9 ± 2.1	NS	10.9 ± 5.2	NS	16.7 ± 6.5	NS
Trienes	3.0 ± 0.3	2.7 ± 0.2	NS	3.1 ± 0.4	NS	2.9 ± 0.8	NS
Tetraenes	9.6 ± 0.8	12.5 ± 0.9	$P < 0.01$	9.5 ± 1.1	NS	13.2 ± 3.3	NS
Polyenes	5.4 ± 2.8	10.9 ± 5.0	NS	14.2 ± 5.8	NS	9.0 ± 4.7	NS

Values are means ± SD (n). Dienes I are monoenoic-monoenoic classes and dienes II are saturated-dienoic classes, respectively. Polyenes include pentaenoic and hexaenoic classes. NS: not significant.

DISCUSSION

This study demonstrated that there are marked differences in the characteristics of phospholipids between human lung carcinoma and normal lung tissue. The main differences were as follows: (a) a marked decrease in the saturated classes, predominantly of a dipalmitoyl species, of PC was noted in the carcinoma as compared with the normal lung tissue, although the human lung carcinoma still contained ca. 17-20% of the saturated classes, and (b) the lung carcinoma contained less

TABLE V

Fatty Acid Composition of Saturated Phosphatidylcholines of Human Lung Carcinoma Isolated by Argentation Thin Layer Chromatography

	Fatty acid composition (mol %)			
	Normal lung	Squamous cell carcinoma	Small cell carcinoma	Adenocarcinoma
14:0	2.4	2.1	3.0	2.5
16:0	92.1	91.6	91.4	90.9
16:1	0.1	0.4	0.3	0.4
18:0	5.1	4.6	4.8	5.6
18:1	0.3	1.0	0.5	0.6

These values were obtained from samples pooled from 3 or 4 cases.

phosphatidylglycerol and lyso-*bis*-phosphatidic acid and more cardiolipin and PI than the normal lung tissue as the acidic phospholipid components. These alterations observed in the lung carcinoma appeared to show that they significantly lose the characteristic feature of phospholipids in lung tissue.

Dipalmitoyl PC is a major surface-active component of pulmonary surfactant, the material which stabilizes the pulmonary alveoli against collapse (32). This compound is the only molecular species which has been specifically implicated in physiological function. However, it has been noted that saturated PC is contained not only in lung tissue, but also in other tissues such as erythrocyte, spleen, kidney, brain (33) and gastric mucosa (34). A previous study (35) demonstrated that rat fetal lung at -4 days, when alveolar Type II cells have not yet appeared, contains ca. 20% of the saturated classes of PC, which seem to be unrelated to the function of surface activity in the lung. These findings indicate that there are at least 2 pools of saturated PC in lung tissue. First, there is the saturated PC synthesized in alveolar Type II cells, the function of which is to supply surface activity in the alveolar space. The other is the saturated PC synthesized in cells other than alveolar Type II cells, which function as the membrane constituents. The lung carcinoma examined in this study were derived from cells other than alveolar Type II cells; hence, they contain the second type of saturated PC, the percentages of which were very consistent with those of rat fetal lung at -4 days (35). These results indicate that, in the content of saturated classes, which contains ca. 30% of the total PC in lung tissue, ca. 20% of that content is in the "membrane pool" whereas the other 10% is in the "surface active pool." One of characteristics of phospholipids in lung carcinoma is the existence of this type of saturated PC. Other carcinoma do not contain such high levels of saturated PC (4).

It is well known that the lung tissue contains, in general, relatively large amounts of phosphatidylglycerol and lyso-*bis*-phosphatidic acid compared to other mammalian tissues (36). It has also been noted that in the lung tissue phosphatidylglycerol may make an important contribution to the surface activity of pulmonary surfactant (37,38), although it may contribute only as a precursor of cardiolipin in other tissues (39). Our previous work (9,40) revealed that phosphatidylglycerol has almost the same profile of molecular species as that of PC in lung tissue, which consists of ca. 20% saturated classes, predominantly of the dipalmitoyl type. The role of alveolar Type II cells or other cells in the synthesis of molecular species of phosphatidylglycerol in the lung remains unknown. However, the marked decrease of phosphatidylglycerol in lung carcinoma may represent the decrease of phosphatidylglycerol in the "surface active pool." Considerable synthesis of phosphatidylglycerol may be carried out in the alveolar Type II cells of the normal lung tissue.

Lyso-*bis*-phosphatidic acid, which was tentatively identified in this study, is known as a lysosomal lipid, which appears in some accumulated diseases such as Nieman-Pick disease (28) and phospholipid liver induced by some drugs (41,42). It has been reported that the lyso-*bis*-phosphatidic acid present in the lung tissue occurs predominantly in macrophages (43). Therefore, it is reasonable that the lung carcinoma not containing macrophages have only a small amount of the phospholipid.

This study also demonstrated the differences in the lipid compositions among different cell types of lung carcinoma. Squamous cell and small cell carcinoma contained more triacylglycerol, as well as relatively higher levels of dienes I (monoenoic-monoenoic) and rather lower levels of dienes II (saturated-dienoic) of

TABLE VI

Positional Distribution of Fatty Acids in Monoenoic Classes of Phosphatidylcholine of Human Lung Carcinoma of Histologically Different Types

	Fatty acid composition (mol %)							
	Normal lung		Squamous cell carcinoma		Small cell carcinoma		Adenocarcinoma	
	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position
16:0	77.1	13.6	76.9	14.6	74.5	17.2	76.8	13.2
16:1	3.4	5.6	4.1	2.2	2.9	1.8	3.3	5.5
18:0	9.1	–	10.2	–	8.6	0.2	9.3	–
18:1	10.1	83.8	8.8	83.2	14.0	80.8	10.6	81.3

Values are means from duplicate analyses.

PC as compared with adenocarcinoma, the lipids of which were similar to normal lung tissue in these points.

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Relationships between Cholesterogenesis, Microsomal Sterols and HMG-CoA Reductase in the Perfused Rat Liver

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ABSTRACT

The relationships between cholesterogenesis and the activity of HMG-CoA reductase of microsomes prepared with or without sodium fluoride, and between changes of cholesterogenesis and microsomal sterols were studied in the isolated rat liver perfused with or without oleic acid in the presence of AY-9944. AY-9944 inhibits the conversion of 7-dehydrocholesterol, measured colorimetrically as "fast-acting" sterols, to cholesterol, measured colorimetrically as "slow-acting" sterols. The level of "fast-acting" sterols is used to estimate cholesterogenesis and changes in microsomal sterols. It was observed that the activity of HMG-CoA reductase of microsomes prepared with or without fluoride reflects the relative changes in cholesterogenesis of the perfused livers. In addition, the amount of "fast-acting" and "slow-acting" sterols in microsomes correlates with increases in the activity of HMG-CoA reductase and cholesterogenesis.

INTRODUCTION

Metabolic inhibitors which block the conversion of sterol intermediates to cholesterol have been used to estimate the synthesis, distribution and metabolism of cholesterol (1-5). One of these inhibitors is AY-9944 [*trans*-1,4-*bis*(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride], which blocks the conversion of 7-dehydrocholesterol to cholesterol (2-4,6). The appearance of 7-dehydrocholesterol, measured colorimetrically as "fast-acting" sterols, has been used extensively to estimate changes in hepatic cholesterogenesis in vivo (7-9). "Fast-acting" sterols refers to sterols which produce maximal color with Liebermann-Burchard reagents within 2 min (10). In contrast, cholesterol is a "slow-acting" sterol which produces maximal color after 30 min. The validity of using the appearance of "fast-acting" sterols after treatment with AY-9944 to estimate the distribution and synthesis of cholesterol in the isolated perfused rat liver was recently demonstrated (11). It was observed that, after 3 hr of perfusion, the amount of "fast-acting" sterols synthesized by the liver correlates with the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), the rate-limiting enzyme of cholesterogenesis. In addition, the amount of "fast-acting" sterols secreted by the perfused liver correlates with the activity of the enzyme and the amount of "fast-acting" sterols synthesized. These relationships were similar to those previously reported to exist between the secretion of cholesterol by the perfused liver and cholesterogenesis estimated either by the activity of the enzyme (12) or by the incorporation of tritium from ^3H_2O into cholesterol (13).

In this study, the appearance of "fast-acting" sterols after treatment of the perfused liver with AY-9944 is used to investigate 2 relationships fundamental to understanding the regulatory mechanism(s) of hepatic cholesterogenesis. The first is the relationship between cholesterogenesis and the activity of HMG-CoA reductase of microsomes isolated from liver homogenized in buffer with or without sodium fluoride. The addition of sodium fluoride to the buffer resulted in a large decrease in the activity of microsomal enzyme. Thus, the physiological significance of estimating cholesterogenesis via HMG-CoA reductase activity from microsomes conventionally isolated without sodium fluoride becomes questionable (14). Brown et al. (15) recently demonstrated that in long-term in vivo studies, the rate of cholesterogenesis correlates with the activity of HMG-CoA reductase of microsomes isolated in the presence, as well as absence, of sodium fluoride. These authors concluded that activity of the enzyme, prepared with or without fluoride, reflects the relative changes in cholesterogenesis which was altered by long-term in vivo treatment. Whether this observation is equally applicable to short-term experiments where cholesterogenesis is rapidly elevated is unknown.

The second relationship investigated in this study is the relationship between changes in microsomal sterols and the activity of microsomal HMG-CoA reductase. Increases in the amount of microsomal cholesteryl esters have been associated with decreases in the activity of HMG-CoA reductase of livers obtained from rats after prolonged feeding of cholesterol (16,17). The decrease in the activity of HMG-CoA reductase, however, can be observed

within 4 hr after the beginning of cholesterol feeding without any changes in microsomal cholesterol (18). Similarly, despite a 100% increase in cholesterogenesis (13) and the activity of HMG-CoA reductase (12), the amount of microsomal cholesterol obtained from rat livers perfused with oleic acid remains the same as those perfused without oleic acid. These observations suggest that in short-term experiments, alterations of microsomal cholesterol, if any, may be very small and difficult to detect. Therefore, investigation of the possibility of using AY-9944 to segregate total microsomal sterols into a newly synthesized pool of cholesterol, "fast-acting" sterols, and a preformed pool of cholesterol, "slow-acting" sterols, in order to study their relationship with cholesterogenesis during perfusion is undertaken.

Observations from this study suggest that increases in cholesterogenesis of the perfused liver can be estimated by the activity of HMG-CoA reductase of microsomes prepared with or without sodium fluoride. In addition, there is a linear relationship between the rate of cholesterogenesis and the increase of microsomal newly synthesized cholesterol and between the rate of cholesterogenesis and the decrease of microsomal preformed cholesterol.

PROCEDURES

Male Sprague Dawley rats from Murphy Breeding Labs Inc., Plainfield, IN, were used in this study. The rats were housed with lighting from 05:00 to 17:00 hours and were fed Wayne Lab-blox and water ad libitum. The livers were removed between 08:50 and 09:50 hours and were perfused in vitro using the apparatus and under the conditions previously described (13,19).

The perfusion medium consisted of Krebs-Ringer bicarbonate buffer, pH 7.4, 100 mg % glucose and 3% of purified bovine serum albumin (20). Following perfusion for a 20-min period of equilibration, a solution of AY-9944 in 0.9% NaCl was added to the perfusate pool to obtain a concentration of 10 μM AY-9944 and infusion into the perfusate was started. The infusate, pH 7.4, contained 0.9% NaCl, 10 μM AY-9944 and 3% purified bovine serum albumin, alone or complexed with 14.16 μmol oleic acid/ml infusate. The infusate was delivered to the perfusate at a constant rate of 11.7 ml/hr during perfusion.

At termination of the experiments, the livers were perfused with a single pass of 20 ml ice-cold 0.9% NaCl and adherent nonhepatic tissue was removed. The liver was then blotted, weighed and minced in an ice-cold beaker. Minced liver tissues were divided into 2 halves and homogenized in 0.3 M sucrose and 10 mM 2-mercaptoethanol with or without sodium fluoride (50 mM) (14). Aliquots were taken for saponification and the remaining portion of liver homogenate was used for the isolation of microsomes as described previously (12). Microsomal pellets isolated from homogenate with or without sodium fluoride were resuspended individually in 50 mM potassium phosphate buffer, pH 7.2, containing 1.0 mM EDTA, 5.0 mM dithiothreitol and 0.3 M potassium chloride. Samples were taken for saponfication and the activity of microsomal HMG-CoA reductase analyzed (21). In brief, microsomes (50-200 μg protein), preincubated for 10 min at 37 C, were incubated for an additional 5-30 min with 200 μM DL [3-^{14}C]-HMG-CoA (6000 dpm/nmol), 5.3 mM NADP, 52 mM glucose-6-phosphate and 1 unit glucose-6-phosphate dehydrogenase in a total vol of 100 μl. The reaction was terminated by the addition of 10 N HCL (25 μl), and nonradioactive mevalonate lactone, 5 mg (25 μl), was added as an internal marker. The mixture, incubated for an additional 30 min, was centrifuged at room temperature. A 50-75 μl aliquot of the supernatant was spotted on the preabsorbant layer of LK5D thin layer plates (Whatman, Clifton, NJ) and developed in benzene/acetone (1:1, v/v). The mevalonate lactone bands were visualized with iodine, recovered and counted.

Samples of liver homogenate, microsomal suspension and perfusate were taken and saponified with 1 ml aqueous KOH (1 g/ml) and 4 ml ethanol for 60 min at 75 C. The nonsaponifiable fractions were extracted into hexane by the addition of 5 ml water and 5 ml hexane followed by vigorous shaking for 10 min. Portions of hexane were removed, dried under N_2 and analyzed colorimetrically for "fast-acting" (2 min) and "slow-acting" sterols (33 min) by the Moore and Baumann procedure (10) with the stable Liebermann-Burchard reagent reported by Kim and Goldberg (22). The amounts of "fast-acting" and "slow-acting" sterols were calculated and corrections made according to the Moore and Baumann procedures (10). The constants used in the calculations described by Moore and Baumann were determined experimentally with 7-dehydrocholesterol (Sigma, St. Louis, MO) and free cholesterol (Nu-Chek-Prep., Elysian, MN).

RESULTS

The effect of oleic acid and sodium fluoride on the activity of microsomal HMG-CoA

TABLE I

Effect of Sodium Fluoride on the Activity of Microsomal HMG-CoA Reductase[a]

Groups	Perfusion time (hr) 1	2	3
	p mol/min/mg protein		
I. Oleic acid omitted			
A: -NaF	38.0 ± 5.0	86.9 ± 2.9	157.4 ± 21.0
B: +NaF	10.7 ± 1.4	15.3 ± 1.5	18.7 ± 2.7
II. Oleic acid added			
C: -NaF	67.4 ± 7.0	127.7 ± 12.0	337.3 ± 48.6
D: +NaF	21.7 ± 1.6	24.5 ± 2.0	38.4 ± 6.2
Statistics[b]:			
A vs C	<0.02	<0.02	<0.01
B vs D	<0.001	<0.01	<0.02

[a]Mean ± SEM; n ≥ 4 for each observation.
[b]Student's t-test.

reductase is shown in Table I. As reported previously by Cooper (23), perfusion of rat liver without oleic acid for 3 hr resulted in an hourly increase in the activity of microsomal HMG-CoA isolated in the absence of sodium fluoride. The hourly increase in the enzyme activity is further enhanced by the addition of oleate to the perfusion medium (A vs C, Table I). The increase in the activity of the enzyme by oleic acid is in agreement with previously reported increases after 4 hr of perfusion (12). The specific activity (sp act) of the enzyme in this study is, however, much less than that of the previous study (12). This discrepancy may be explained by the low activity level of microsomal HMG-CoA reductase in the rats used in this study. Without treatment with sodium fluoride, the activity of microsomal HMG-CoA reductase is 39 ± 6.0 pmol/min/mg (n = 5) for livers that were not perfused. This value is comparable to the diurnal low for the activity of the enzyme reported by some (e.g., 24,25) and lower than others (e.g., 15). The differences are not readily apparent. Factors which may be responsible for this difference include strains of rats, housing conditions, feeding pattern, weight of animals, diet and conditions for the isolation and analysis of the microsomal activity of HMG-CoA reductase. Regardless, a 114% increase in the activity of the enzyme by oleate in these experiments using 3 hr of perfusion is comparable to the 140% increase after 4 hr of perfusion reported earlier (12). Also shown in Table I is the reduction of HMG-GoA reductase activity by the addition of sodium fluoride to the homogenization medium. Sodium fluoride did not alter the apparent increase in the activity of microsomal HMG-CoA reductase from liver perfused with oleate (B vs D, Table I). The effect of sodium fluoride on the relationship between the activity of microsomal HMG-CoA reductase and cholesterogenesis, estimated by the synthesis of "fast-acting" sterols is shown in Figure 1. The synthesis of "fast-acting" sterols is the sum of "fast-acting" sterols in the perfusate at the end of the experiment and the amount in the liver homogenized in buffer without sodium fluoride. The amount of "fast-acting" sterols in livers homogenized with or without sodium fluoride is the same, and the value obtained

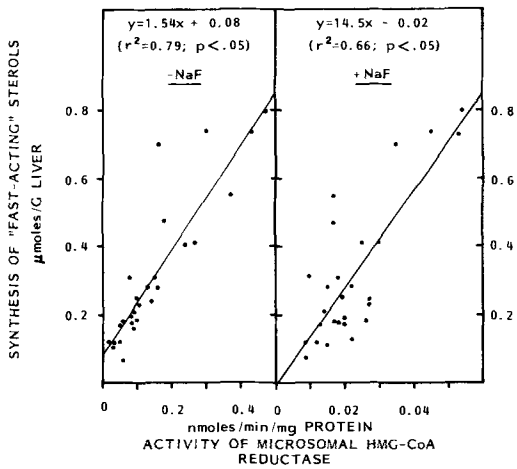

FIG. 1. Correlation between the synthesis of "fast-acting" sterols and the activity of microsomal HMG-CoA reductase. Data for HMG-CoA reductase represents the individual experiments reported in Table I. The synthesis of "fast-acting" sterols is the sum of "fast-acting" sterols in the perfusate at the end of the perfusion and the amount in the liver determined in portions of the liver homogenized in buffer without sodium fluoride.

from liver homogenate without sodium fluoride is used in calculating the synthesis of "fast-acting" sterols. As is shown in Figure 1, the activity of HMG-CoA reductase of microsomes isolated in the absence of sodium fluoride correlates linearly with cholesterogenesis with a slope parallel to that of microsomes isolated in the presence of sodium fluoride.

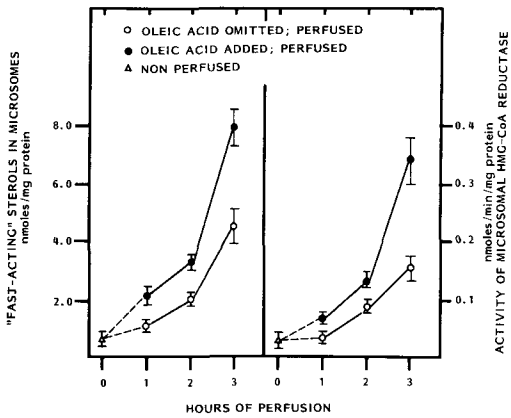

FIG. 2. Effect of oleic acid on microsomal "fast-acting" sterols and the activity of HMG-CoA reductase. The data shown are means ± SEM. All comparisons between groups with or without oleate are significant ($P < 0.05$) by Student's t-test. Each data point represents $n \geq 4$. Values for nonperfused livers were obtained from hepatic microsomes of rats sacrificed at 09:00. The amounts of microsomal "fast-acting" sterols in livers perfused 3 hr without AY-9944 are 0.64 ± and 0.57 ± 0.1 (n = 5) nmol/mg protein with and without oleic acid, respectively. Values shown were obtained from liver homogenate and microsomes without treatment with sodium fluoride.

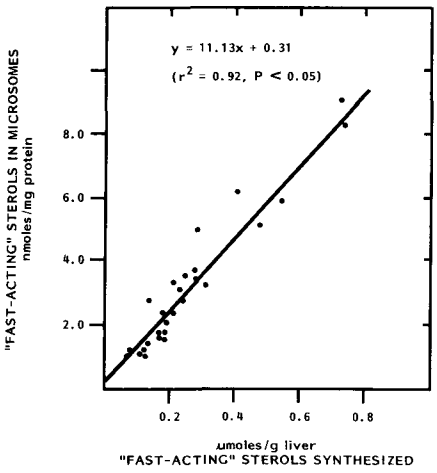

FIG. 3. Relationship between the synthesis and the concentration of "fast-acting" sterols in microsomes. The amount of "fast-acting" sterols is obtained from microsomes isolated in the absence of sodium fluoride. Additional details are given in the legend of Figure 1.

Changes in the amount of "fast-acting" sterols in microsomes obtained from the perfused liver homogenized without fluoride is shown in Figure 2 (left panel). The amount of microsomal "fast-acting" sterols is elevated by the length of perfusion and further enhanced by oleate. The pattern of increases in microsomal "fast-acting" sterols mimics that of the secretion of "fast-acting" sterols by the liver into the perfusate (11) and the activity of HMG-CoA reductase in microsomes isolated in the presence (right panel, Fig. 2) or absence (figure not shown) of sodium fluoride. Increases in the amount of microsomal "fast-acting" sterols apparently are synchronous with and related to the activity of the enzyme isolated with or without sodium fluoride. For example, the linear relationship between changes in the amount of microsomal "fast-acting" sterols (y) in nmol/mg protein, and the activity of HMG-CoA reductase from microsomes isolated without sodium fluoride (x), in pmol/min/mg protein can be described by the equation $y = 0.02x + 1.03$ ($r^2 = 0.81$; $P < 0.05$) (figure not shown). In addition, the changes in microsomal "fast-acting" sterols correlate linearly with the synthesis of "fast-acting" sterols by the perfused rat liver (Fig. 3).

Changes in the level of microsomal "slow-acting" sterols by perfusion and by oleic acid are shown in Table II. "Slow-acting" sterols represent the free and esterified cholesterol already present in the microsomes or liver before perfusion in the presence of AY-9944. There is a general decrease in microsomal "slow-acting" sterols as perfusion of the liver continues from 1 to 3 hr. Oleate did not significantly alter the decrease in the amount of microsomal "slow-acting" sterols (A vs B, Table II), in contrast to its effect on microsomal "fast-acting" sterols (Fig. 2). On the other hand, when the changes in microsomal "slow-acting" sterols are calculated as a percentage of total microsomal sterols, the sum of "slow" and "fast-acting" sterols, the decrease in microsomal "slow-acting" sterols becomes apparent (C vs D, Table II). In addition, the decrease in microsomal "slow-acting" sterols, calculated in this way, correlates inversely with the increase in the activity of microsomal HMG-CoA reductase (Fig. 4).

DISCUSSION

AY-9944, which inhibits the conversion of 7-dehydrocholesterol to cholesterol (2-4), is used with the isolated rat liver perfusion model to study factors associated with changes in

TABLE II

Effect of Oleic Acid on the Changes of "Slow-acting" Sterols in Microsomes[a]

Oleic acid in perfusate	Perfusion time (hr)		
	1	2	3
		nmoles/mg protein	
A: Omitted	70.2 ± 5.7	49.2 ± 2.3	45.0 ± 2.9
B: Added	66.6 ± 2.0	62.7 ± 7.8	43.4 ± 1.4
		total sterols[b] (%)	
C: Omitted	98.6 ± 0.1	96.3 ± 0.2	90.6 ± 1.2
D: Added	97.4 ± 0.3	94.2 ± 0.7	84.7 ± 1.0
Statistics[c]:			
A vs B	<0.70	<0.80	<0.70
C vs D	<0.025	<0.05	<0.02

[a]Mean ± SEM; n ⩾ 4 for each observation obtained from microsomes prepared without fluoride.

[b]("Slow-Acting" sterols X 100)/("Slow-Acting" + "Fast-Acting" Sterols).

[c]Student's t-test.

cholesterogenesis during short-term perfusion. 7-Dehydrocholesterol accumulated during treatment with AY-9944 is measured as newly synthesized "fast-acting" sterols (10) and is distinguished from preformed "slow-acting" cholesterol. Cholesterogenesis of the perfused liver was elevated by perfusion for various periods of time and with oleic acid. These treatments led to an increase in the activity of microsomal HMG-CoA reductase (12,26), cholesterogenesis estimated by the incorporation of tritium (^3H_2O) into cholesterol (13) and the secretion of cholesterol by the liver (12,13).

The first factor studied is the relationship between cholesterogenesis and the activity of HMG-CoA reductase of microsomes prepared with or without sodium fluoride. The in vivo long-term study by Brown et al. (15) demonstrated the validity of using the HMG-CoA reductase activity of microsomes prepared with or without sodium fluoride to reflect the relative changes in cholesterogenesis. The synthesis of cholesterol in their study was determined by the incorporation of [1-^{14}C]-octanoate into cholesterol during incubation of liver slices. In this study, cholesterogenesis is measured by the amount of "fast-acting" sterols synthesized during perfusion. Linear correlation between the synthesis of "fast-acting" sterols and activity of HMG-CoA reductase of microsomes prepared with or without fluoride (Fig. 1) suggests that enzyme activity is a valid estimation of the relative changes in cholesterogenesis regardless of the presence or absence of fluoride in the homogenization buffer. Thus, the activity of HMG-CoA reductase of microsomes prepared either with or without sodium fluoride reflects relative changes in hepatic cholesterogenesis under conditions where synthesis of cholesterol was modified by long-term in vivo treatment (15) or by rapid short-term in vitro measures such as perfusion with oleic acid.

The second factor studied is the relationship between changes in microsomal sterols and rate of cholesterogenesis. The changes in total microsomal sterols are determined by the amount of "fast-acting" sterols, representing newly synthesized cholesterol, and "slow-acting" sterols, representing preformed cholesterol. Small and significant changes in "fast-acting" sterols can be detected in microsomes

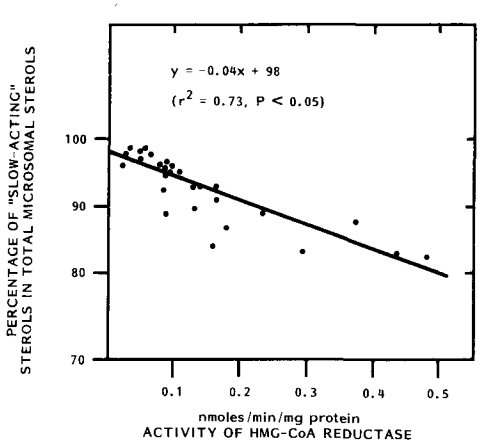

FIG. 4. Relationship between changes in microsomal sterols and the activity of HMG-CoA reductase. The data shown are the individual experiments reported in this manuscript and additional information is given in the legend to Table II.

after perfusion for 1 hr with oleate (Fig. 2). The increase in the amount of "fast-acting" sterols in microsomes appears to be a specific process. This is implicated by the correlation between the amount of microsomal "fast-acting" sterols and the activity of microsomal HMG-CoA reductase as well as the synthesis of "fast-acting" sterols by the liver (Fig. 3). Thus, the amount of newly synthesized cholesterol appearing in the microsomes depends on the rate of synthesis of cholesterol by the microsomes.

Concurrently, with the increase of newly synthesized cholesterol in microsomes, preformed "slow-acting" cholesterol in microsomes is reduced when changes in "slow-acting" sterols are calculated as a percentage of total microsomal sterols (Table II). Biological variations in the amount of microsomal cholesterol, "slow-acting" sterols, before livers were perfused may account for the less obvious decline of "slow-acting" cholesterol when calculated in nmol/mg microsomal protein (Table II). The correlation between the percentage decrease in "slow-acting" sterols with cholesterogenesis (Fig. 4) suggests that the removal of preformed microsomal cholesterol can be related to the increase in the synthesis of cholesterol.

The relationship between changes in microsomal sterols and cholesterogenesis has important implications in the study of regulatory mechanism(s) of hepatic cholesterogenesis. The mechanism(s) regulating cholesterogenesis at the molecular level is unknown and several hypotheses have been suggested. Of these, regulation by changes in microsomal fluidity (27) and regulation by direct end-product inhibition (28) have been related to changes in microsomal sterols. The regulation by end-product inhibition hypothesis suggests an inverse relationship between changes in microsomal sterols and the rate of cholesterogenesis mediated, presumably, through microsomal HMG-CoA reductase. Evidence for the decrease in the activity of HMG-CoA reductase and a concomitant increase in microsomal sterols has been reported (16,17). Evidence for the converse, an increase in the activity of HMG-CoA reductase and a decrease in microsomal sterols is unavailable. It can, however, be inferred from the increase in the activity of the enzyme and the increased efflux of cholesterol from the liver (12,29) and hepatocytes (30-32). The correlation between the decrease in preformed microsomal "slow-acting" sterols and increased cholesterogenesis (Fig. 4) demonstrates an inverse relationship between cholesterogenesis and the removal of microsomal preformed cholesterol. This, together with the decrease of enzyme activity caused by the elevation of microsomal sterols (16,17), supports the hypothesis of direct end-product inhibition as the regulatory mechanism for hepatic cholesterogenesis. Regardless of the molecular mechanism involved, data from the study on the relationship between changes in microsomal sterols and cholesterogenesis indicate that the removal of preformed cholesterol from microsomes may be the immediate stimulus for increasing the synthesis of cholesterol. This removal results in the appearance of newly synthesized cholesterol, "fast-acting" sterols, in microsomes.

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Hepatic Δ^9 and Δ^6 Desaturase Activities during the Recovery Period Following Carbon Tetrachloride Poisoning

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ABSTRACT

The liver microsomal Δ^9 and Δ^6 desaturase activities have been studied in rats with carbon tetrachloride-induced hepatitis. Immediately after poisoning, significant decreases were observed for both types of desaturase activity. However, recovery kinetics were slower for the Δ^6 desaturase than for the Δ^9 desaturase. The activities of NADH-ferricyanide and NADH-cytochrome C reductases, proteins involved in the electron transfers associated with microsomal desaturation, were also measured. There was a fall in both activities after poisoning, but this decrease was less than that of the desaturase activities.

INTRODUCTION

Some insight into the process of liver regeneration may be gained from a sequential study of the activities of certain enzymes after injury by hepatocellular poisoning (1-3). During tissue regeneration, the unsaturated fatty acids produced by acyl-CoA desaturases are among the essential components of cell membranes to be rebuilt. Therefore, an investigation of the liver microsomal desaturation system after poison ingestion could contribute to the analysis of the recovery process.

The microsomal acyl-CoA desaturase systems involve several proteins. For instance, the system which desaturates stearyl-CoA into oleyl-CoA is composed of 3 different proteins (4,5): NADH-cytochrome b5 reductase (6-8), cytochrome b5 (9,10) and the desaturase itself (11,12). Cytochrome b5 also is involved in the desaturation of linoleate into γ-linolenate (13,14). As is the case with all membrane enzymes, the activities of the enzymes in the acyl-CoA desaturase systems could be controlled by the lipid environment of the active sites of the proteins (4,15,16).

In this report, we describe the variations of the activities of the Δ^9 and Δ^6 desaturases from rat liver microsomes during the regeneration period following CCl_4 poisoning, The levels of microsomal NADH-ferricyanide and NADH-cytochrome C reductase have also been measured; their activities are coupled to NADH-cytochrome b5 reductase and to the reduction of cytochrome b5, respectively. The measurement of these enzymes would enable us to follow the different components of several microsomal desaturase systems during the regeneration process.

MATERIAL AND METHODS

Treatment of Animals

Five- to 6-wk-old male rats (Wistar CF), weighing 110-120 g, were maintained on a commercial lab chow and ad libitum water. Carbon tetrachloride (Merck Chem., Darmstadt, Germany) diluted in liquid paraffin (1:1,v/v) was administered intragastrically. The dose of the carbon tetrachloride given was 150 $\mu\ell$ of CCl_4/100 g body weight diluted in 350 $\mu\ell$ of liquid paraffin. The activity of the desaturases was measured at 3, 6 and 12 hr as well as 1, 2, 4, 7 and 14 days postingestion of poison. In each experiment, control animals were given 500 $\mu\ell$ of liquid paraffin.

Chemicals

[1^{14}-C] Stearic acid (50 mCi/mmol) and [1^{14}-C] linoleic acid (50 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Corresponding acyl-coenzyme A esters were prepared according to the Ailhaud and Vagelos procedure (17) as modified by Bourre and Daudu (18). NADH was purchased from Sigma (St. Louis, MO).

Preparation of Microsomes

The liver was rapidly removed from rats killed by decapitation, weighed and homogenized in 3 vol (v/w) of ice-cold solution of 250 mM sucrose. A postmitochondrial supernatant fraction, obtained by centrifugation at 15,000 x g for 30 min, was further centrifuged at 100,000 x g for 60 min to give the microsomal pellet. This pellet was suspended in 0.5 vol (v/w) 250 mM sucrose solution. Protein concentration was determined according to Lowry et al. (19).

Enzyme Assays

Acyl-CoA desaturases. The final incubation mixture (1 ml) contained 0.1 M sodium phosphate (pH 7.4), 1 mM NADH, 50 nmol [1^{14}-C]-acyl-CoA (120,000 cpm) and 1.0 mg microsomal protein (20). Prior to the addition of microsomes, the assay medium was aerated by bubbling with oxygen for 10 sec. The incubations were performed in a thermoregulated shaking water bath at 37 C for 20 min.

The reaction was terminated by the addition of 1.0 ml of 10% methanolic KOH and heating at 80 C for 30 min. The fatty acids were liberated by the addition of 1.0 ml 7 N HCl and heating at 80 C for 10 min. Fatty acids were extracted twice by 2 ml *n*-hexane/extraction. Fatty acid methyl esters were prepared with N methanolic anhydrous HCl, as described by Carreau and Dubacq (21). Radiolabeled methyl esters were then analyzed either by radio gas chromatography (radio-GC) (Packard Instrument, series 894) or measured with a liquid scintillation spectrometer (Intertechnique, Series SL 30) after thin layer chromatography (TLC) on silver nitrate-impregnated Silica Gel G plates. Commercial Silica Gel G- (Merck) impregnated plates were immersed for 10 sec in a 80% ethanol solution containing 10% silver nitrate. Benzene/hexane (7:3, v/v) was used as the developing solvent to separate monounsaturated and saturated fatty acid methyl esters (22). To separate linoleic and γ-linolenic acid methyl esters, a mixture of benzene/hexane/diethyl ether (7:3:0.75, v/v/v) was used. The spots were revealed by rapid immersion of the plates in 0.1% 1.7-dichlorofluorescein solution and examination under ultraviolet (UV) light. The marked spots were scraped off and coated silica gel was placed in 15 ml of a toluene solution containing PPO and POPOP.

Reductases. Activities were measured according to the Lee et al procedures (23) for NADH-ferricyanide reductase and the Sottocasa et al. method (24) for NADH-cytochrome C reductase.

Acyl-CoA hydrolysis and fatty acids incorporation into complex lipids. CoA esters were incubated under the same conditions as those used for the assay of desaturase activities. Following an incubation of 20 min, the reaction was terminated by the addition of 1.0 ml methanol and 2.0 ml chloroform. Lipids were chromatographed on thin layer silica gels. Free fatty acids and triglycerides were separated according to Brown and Johnston (25) and phosphatidylcholine (PC) according to Katyal et al. (26). The marked spots were scraped off and the coated silica gel was placed in 15 ml of a toluene solution containing PPO and POPOP.

RESULTS

Δ^9 Desaturase Activity

Six hr after CCl_4 ingestion stearyl-CoA desaturase activity in liver microsomes from poisoned rats was decreased (Table I). The lowest values were observed between 12 and 24 hr: 25-30% of control values. After 48 hr, Δ^9 desaturase activity increased again but a return to normal values still was not complete after 14 days.

Δ^6 Desaturase Activity

The activity of linoleyl-CoA desaturase was also affected by CCl_4 poisoning and a decrease was observed 3 hr after ingestion with maximal effect at 12 hr (Table I). A progressive restoration process began at 24 hr but was slower than that of Δ^9 desaturase activity since at 14 days the Δ^6 desaturase activity remained at 70-75% of controls.

NADH-ferricyanide Reductase and NADH-cytochrome C reductase Activities

The NADH-ferricyanide reductase activity of liver microsomes from rats having ingested 150 $\mu\ell$ of CCl_4/100 g body weight decreased rather slowly after poisoning (Table II). By 12 hr after CCl_4 ingestion, there was a drop of 15% in the NADH-ferricyanide reductase activity. This decrease reached 40% by 24 hr, which was then followed by a gradual increase to normal values by the 7th day.

Three to 6 hr after CCl_4 ingestion, the NADH-cytochrome C reductase activity in liver microsomes from poisoned rats was higher than that of control animals (Table II). Thereafter a decrease occurred but, in comparison to that of NADH-ferricyanide reductase, was less immediate and became obvious only 48 hr postingestion.

Fatty Acid Composition of the Total Lipid of Liver Microsomes

As a consequence of CCl_4 poisoning, the content of palmitic acid in liver microsomes increased from the 12th hr through the 4th day (Table III). During this same period, the content of arachidonic and stearic acids decreased but that of linoleic acid remained slightly higher than that of control rats.

Hydrolysis of Acyl-CoA and Incorporation of Precursors into Complex Lipids

The influence of CCl_4 on the hydrolytic

TABLE I

Δ^9 and Δ^6 Desaturation Activities during the Recovery Period following Carbon Tetrachloride Poisoning

Enzyme assay		Time after CCl_4 ingestion							
		3 hr	6 hr	12 hr	1 day	2 days	4 days	7 days	14 days
Stearyl-CoA desaturation[a]	Controls[b]	10.3 ± 0.6	9.8 ± 0.4	11.3 ± 0.9	10.2 ± 0.7	11.5 ± 0.8	9.7 ± 0.3	10.2 ± 1.1	9.7 ± 0.2
	CCl_4[c] treated	10.0 ± 0.2	6.2 ± 0.04	2.8 ± 0.3	3.0 ± 0.4	4.8 ± 0.7	5.4 ± 0.5	8.2 ± 0.7	9.1 ± 0.5
	Relative[d] activity	98.1	63.5	24.1	29.2	42.1	55.9	80.1	94.1
Linoleyl-CoA desaturation[e]	Controls	8.2 ± 0.5	9.0 ± 0.7	7.8 ± 0.7	8.0 ± 0.5	8.9 ± 0.2	8.2 ± 0.3	7.6 ± 0.3	9.3 ± 0.3
	CCl_4-treated	5.7 ± 0.5	6.0 ± 1.0	2.9 ± 0.6	3.6 ± 0.6	4.5 ± 0.4	4.4 ± 0.9	5.2 ± 0.4	6.7 ± 0.7
	Relative activity	69.5	66.1	37.4	45.0	50.0	53.5	69.2	72.3

[a]nmol Oleic acid formed/mg protein/20 min.

[b]Controls animals were given 500 µl of liquid paraffin.

[c]Experiment animals were given of 150 µl of CCl_4 in 350 µl of liquid paraffin per 100 g of body weight.

[d]As percentage of controls.

[e]nmol γ-Linoleic acid formed/mg protein/20 min.

Each value represents the mean ± SD of 3 independent determinations; the liver microsomes of 3 different rats were used in duplicate experiments.

TABLE II

NADH-ferricyanide Reductase and NADH-cytochrome C Reductase Activities after Carbon Tetrachloride Poisoning

Enzyme assay		Time after CCl_4 ingestion							
		3 hr	6 hr	12 hr	1 day	2 days	4 days	7 days	14 days
NADH-ferricyanide reductase activity[a]	Controls[b]	3.86 ± 0.06	3.56 ± 0.04	3.71 ± 0.45	3.58 ± 0.35	3.21 ± 0.36	3.10 ± 0.48	3.26 ± 0.35	3.42 ± 0.43
	CCl_4-treated[c]	3.72 ± 0.16	3.88 ± 0.18	3.12 ± 0.18	2.09 ± 0.26	2.30 ± 0.20	2.39 ± 0.10	3.10 ± 0.26	3.33 ± 0.31
NADH-cytochrome C reductase activity[d]	Controls	1.24 ± 0.02	1.03 ± 0.07	1.03 ± 0.05	0.99 ± 0.07	0.90 ± 0.09	0.97 ± 0.09	0.94 ± 0.11	1.03 ± 0.05
	CCl_4-treated	1.54 ± 0.12	1.68 ± 0.06	1.07 ± 0.06	0.99 ± 0.05	0.66 ± 0.06	0.64 ± 0.05	0.79 ± 0.06	0.90 ± 0.18

[a]µmol Ferricyanide reduced/mg protein/min.

[b]Control animals were given of 500 µl of liquid paraffin.

[c]Experimental animals were given of 150 µl of CCl_4 in 350 µl of liquid paraffin per 100 g of body weight.

[d]µmol Cytochrome C reduced/mg protein/min.

Each value represents the mean ±SD of 2 measurements carried out in duplicate on microsomal preparations from 2 livers.

TABLE III

Fatty Acid Composition of the Lipids Extracted from Liver Microsomes of Carbon Tetrachloride-treated Rats

Time after CCl_4 treatment	Fatty acids					
	16:0	16:1	18:0	18:1	18:2	20:4
controls[a]	23.8 ± 1.2	1.4 ± 0.9	24.0 ± 2.0	11.3 ± 0.7	15.7 ± 2.0	23.6 ± 1.2
3 hr[b]	25.9 ± 0.6	1.1 ± 0.5	26.5 ± 1.0	10.7 ± 0.6	15.1 ± 0.2	20.2 ± 1.1[c]
6 hr	25.8 ± 1.1	1.9 ± 0.3	26.4 ± 1.4	10.9 ± 0.1	15.3 ± 0.9	19.6 ± 0.6[d]
12 hr	28.4 ± 2.0	1.7 ± 0.2	23.8 ± 0.5	11.6 ± 0.9	18.4 ± 0.3[c]	16.4 ± 0.4[d]
1 day	29.4 ± 1.0[c]	2.7 ± 0.5	21.7 ± 0.1[c]	10.3 ± 0.1[c]	18.6 ± 1.0[c]	17.0 ± 0.2[e]
2 day	29.1 ± 3.0	1.2 ± 0.4	21.5 ± 2.0	9.4 ± 0.8	17.3 ± 0.9	18.9 ± 0.4[d]
4 day	25.5 ± 1.7	1.2 ± 0.3	21.2 ± 0.8[c]	13.3 ± 1.8	14.4 ± 1.0	25.6 ± 0.3[c]
7 day	23.2 ± 2.2	1.2 ± 0.1	26.4 ± 1.1	9.1 ± 0.2[d]	13.6 ± 0.2	26.4 ± 1.7[c]
14 day	24.8 ± 1.7	1.1 ± 0.1	25.1 ± 1.1	9.3 ± 0.6	16.4 ± 0.1	23.1 ± 0.1

[a]Each value is the mean ± SD of 5 independent determinations.

[b]Each value is the mean ± SD of 3 independent determinations.

[c]Differences significant at $P < 0.05$.

[d]Differences significant at $P < 0.01$.

[e]Differences significant at $P < 0.001$.

Two-tailed t-test values calcd according to Cochran (27).

TABLE IV

Hydrolysis of Acyl-CoA and Fatty Acids Incorporation into Triglycerides and Phosphatidylcholine (PC) by Liver Microsomes from Carbon Tetrachloride-treated Rats

	Controls	3 hr	6 hr	12 hr	1 day	2 days	4 days	7 days	14 days
Free stearic acid	5.25 ± 0.71[a]	7.10 ± 0.81	8.77 ± 1.20	8.35 ± 0.83	7.40 ± 0.61	7.96 ± 0.24	7.05 ± 0.41	6.21 ± 0.47	6.17 ± 0.16
Stearic acid incorporated into triglycerides	0.98 ± 0.32[b]	1.64 ± 0.25	1.54 ± 0.17	1.77 ± 0.32	1.44 ± 0.38	0.90 ± 0.47	0.81 ± 0.20	1.01 ± 0.17	1.17 ± 0.25
Stearic acid incorporated into PC	4.65 ± 0.91[c]	4.35 ± 0.85	4.05 ± 0.52	4.25 ± 0.52	4.55 ± 0.55	5.40 ± 0.71	5.35 ± 0.65	5.47 ± 0.69	4.81 ± 0.34
Free linoleic acid	7.20 ± 0.11[a]	7.82 ± 0.37	9.35 ± 1.31	9.15 ± 1.00	9.70 ± 0.81	9.75 ± 0.25	8.70 ± 1.05	8.00 ± 0.22	7.40 ± 0.69
Linoleic acid incorporated into triglycerides	1.25 ± 0.28[b]	1.15 ± 0.15	1.10 ± 0.30	1.45 ± 0.16	1.31 ± 0.42	1.35 ± 0.28	1.01 ± 0.36	1.30 ± 0.11	0.98 ± 0.25
Linoleic acid incorporated into PC	5.35 ± 0.83[c]	6.00 ± 0.55	4.85 ± 0.21	4.80 ± 0.80	4.12 ± 0.29	5.06 ± 0.39	5.86 ± 0.41	5.85 ± 0.62	6.28 ± 0.72

[a]nmol Stearic or linoleic acids released/mg protein/20 min.

[b]nmol Stearic or linoleic acids incorporated/mg protein/20 min.

[c]nmol Stearic or linoleic acids incorporated/mg protein/20 min.

Each value represents the mean ± SD of 2 independent determinations on liver microsomes of 2 different rats.

activity of the Acyl-CoA was observed by 3 hr postingestion (Table IV); this increase persisted for 7 days.

Carbon tetrachloride enhanced the incorporation of stearic acid into triglycerides during the 1st day postingestion. No changes were observed in incorporation of linoleic acid into triglycerides. Incorporation of both stearic and linoleic acids into phosphorylcholine was unaffected by CCl_4 treatment.

Food Intake

Control rats ate 14.8 g ± 1.8/100 g body weight of Purina chow/day. The food intake of poisoned rats was 12.1 g ± 1.1, 7.8 g ± 1.8 and 14.0 g ± 2.3 (per 100 g body weight) at the 1st, 2nd, 4th and 7th days post-CCl_4-treatment, respectively.

DISCUSSION

Carbon tetrachloride poisoning induces strong perturbations in the liver microsomal desaturation systems. This perturbation is reflected by a drop in the microsomal acyl-CoA desaturase activities within 12 hr. At this time, the decreases for stearyl-CoA and linoleyl-CoA desaturase activities are 75 and 65%, respectively, of normal activities. As the effect of CCl_4 subsides, all desaturase activities are regained although at different rates. Recovery of the Δ^6 desaturation system is slower, its activity remaining at 70-75% that of controls 14 days after poison ingestion. These results are compatible with the finding that the Δ^6 and Δ^9 desaturase systems have different regulatory mechanisms (28).

The decrease in activity of microsomal desaturases following CCl_4 ingestion may be the result of the natural disappearance of enzymes which have short half-lives, e.g., 3-4 hr for the Δ^9 desaturase (29), but is more likely to be the consequence of the disorganization of the microsomal membranes. However, the precise mechanism of membrane disorganization is unknown (30) and the targets of CCl_4 molecules are yet to be discovered. Following toxic injury, the membrane recovery process apparently follows 2 modes (31). In the first, formation of new membranes occurs entirely de novo; in the second, membranes could be rebuilt from remnants of former membranes having reoccupied their previous sites in the cell structure. Following poison ingestion, both processes could coexist during the first 24 or 48 hr, thus explaining the high rate of recovery of enzyme activity exhibited during this period.

In the situation under study, the alimentary contribution apparently does not play a role, since the maximal decrease in Δ^9 desaturase recovery had occurred prior to a significant fall in food intake.

After CCl_4 poisoning, both NADH-ferricyanide and NADH-cytochrome C reductase activities decrease moderately. Seven days after poison ingestion, the new microsomal membranes apparently already possess their full complement of flavoproteins (NADH-ferricyanide reductase) and of cytochrome b5 (NADH-cytochrome C reductase). These data are consistent with other evidence that NADH-cytochrome C and NADH-ferricyanide reductases do not intervene in the regulation of the Δ^9 and Δ^6 desaturase activities (32).

In this experiment, the perturbation of the Δ^9 desaturase activity does not modify the oleic acid content of liver microsomes which remain at values close to those of control microsomes. When Δ^9 desaturase is at its minimum, stearic acid is at a minimum and palmitic acid is at a maximum. This would indicate that CCl_4 has a marked effect on the elongation of palmitic acid. This may be a direct or indirect effect, i.e., direct action on the elongase, or indirect, caused by the inhibition of the Δ^9-desaturase. Linoleyl-CoA desaturase is the rate-limiting step in the metabolic pathway leading to arachidonic acid (33). In accordance with Horning et al. (34), we have found that CCl_4 poisoning induces a slight increase in linoleic acid and a significant drop in arachidonic acid content in liver microsomes. These shifts occur subsequently to the decrease in Δ^6 desaturase activity. The increase in arachidonic acid content observed by the 4th day post-CCl_4 poisoning is more likely related to the contribution of circulating lipids than to microsomal synthesis from linoleic acid.

The hydrolysis of acyl-CoA and the incorporation of fatty acids into triglycerides are reactions which compete with oxidative desaturation for the use of acyl-CoA. In agreement with the work of Shimizu (35), we also have observed that CCl_4 increases the activity of acyl-CoA hydrolase and enhances the incorporation of stearic acid into triglycerides. When compared to liver microsomes from normal rats, the changes observed in the microsomes of CCl_4-treated rats seem too small to account for the variations in Δ^9 and Δ^6 desaturase activities.

We therefore assume that the perturbations of the Δ^9 and Δ^6 desaturase activities measured in liver microsomes of CCl_4-treated rats bear upon the terminal cytochrome b5 oxidase of the system desaturating fatty acids. During the liver regeneration which follows CCl_4 poisoning, the terminal factor involved in Δ^6

desaturation is restored more slowly than the one involved in Δ^9 desaturation.

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Lipoprotein Lipid and Protein Synthesis in Experimental Nephrosis and Plasmapheresis: II. Perfused Rat Liver

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ABSTRACT

Livers from rats with experimental hypoproteinemia induced by aminonucleoside-nephrosis or plasmapheresis were perfused with a [^{14}C]-labeled amino acid mixture at physiological concentration. Compared to control rats, a significantly increased incorporation of the amino acid label was found in the apolipoproteins of the ultracentrifugally separated very low and high density lipoproteins (VLDL, HDL), and into albumin secreted into the perfusate. However, no increase in the amino acid-derived label was detected in VLDL- or HDL-borne lipids in nephrosis or plasmapheresis. Perfusion with U-[^{14}C] leucine as a lipogenesis precursor at >10 times higher than physiological concentration resulted in 5-fold increase in the label incorporation into perfusate proteins in nephrosis but only in a slightly significant increase in perfusate lipids. In contrast, the incorporation of a preformed fatty acid, 9,10-[^{3}H] oleate into VLDL and HDL lipids increased 3- to 4-fold in nephrosis. Both with leucine and oleate as precursors, the increments in the label appearing in perfusate proteins or lipids, respectively, were markedly greater than the increases in hepatic tissue proteins or lipids. The results indicate that amino acids are preferentially directed by the liver into the synthesis of circulating apolipoproteins and albumin in hypoproteinemia and do not seem to constitute an important precursor of the lipoprotein lipids. The increased production of apolipoproteins is associated with an increased incorporation of preformed fatty acids into lipoprotein lipids in addition to the previously reported stimulation of hepatic de novo lipid synthesis from precursors other than amino acids.

INTRODUCTION

In a preceding paper (1), an increase in synthesis of lipoprotein-borne lipids using [^{14}C] citrate or $^{3}H_2O$ as precursors was demonstrated in intact rats with hypoalbuminemia resulting from nephrosis or plasmapheresis, together with an increased flow of [^{3}H] leucine into plasma apolipoproteins and albumin. On the other hand, the enhanced synthesis of lipoproteins was not associated with appreciable incorporation of leucine label into the lipids, although leucine is known to be a precursor of acetyl-CoA in the liver (2) and is readily converted into lipids by adipose tissue (3). In this study, we wished to obtain better insight into the origin of lipoprotein lipids in nephrosis and plasmapheresis using the isolated perfused liver preparation and [^{14}C] labeled amino acids. The question of interest was whether amino acids, at physiological concentration or in excess, would serve as a common precursors for the enhanced synthesis of both lipid and protein moieties of the lipoproteins. Information was also sought on the use of preformed fatty acids as a possible lipid source in response to the increased availability of apolipoproteins in nephrosis.

EXPERIMENTAL PROCEDURES

Animals

Male ad libitum-fed albino rats of Hebrew University strain, weighing 240-260 g were used. Nephrosis was induced by 6-7 daily injections of 2.0 mg/100 g aminonucleoside of puromycin (Sigma, St. Louis, MO) and the rats were used 7-10 days after the last injection. Plasmapheresis was performed by 2 withdrawals of .25 of blood vol at 18-hr intervals by cardiac puncture, with an immediate reinjection of an equivalent amount of homologous red blood cells in 0.9% NaCl; the rats were used for experiments 3 hr after the last plasma removal. Further details on the experimental animals are given elsewhere (1).

Liver Perfusion

The liver was perfused in a Harvard apparatus (I.R. Thebeau, Boston, MA) essentially according to the Miller et al. procedure (4). The gas mixture was 95% O_2, 5% CO_2. The perfusate medium was a Krebs-Ringer bicarbonate buffer, pH 7.4, which contained 5% bovine free fatty acid-(FFA) poor serum albumin, 0.1% glucose, 20% fresh, washed human erythrocytes, 5 units/ml heparin, an amino acid mixture at physiological concentration according to Li et al. (5) and streptomycin and penicillin according to Tracht et al. (6). The total perfusate vol was 130 ml. The perfusion flow rate was 15 to 20 ml/min at a hydrostatic pressure of 18-20 cm. Liver function throughout the perfusion was monitored by checking the perfusate pH (7.35-7.45), lactate/pyruvate ratio

(10-20), linearity of urea production (ca. 1 mg/hr/g) and the general appearance of the liver.

Several experiments were performed using homologous albumin, isolated from rat serum by precipitation of globulins at 50% $(NH_4)_2SO_4$ concentration and dialysis, and rat blood cells. The liver function tests and amino acid incorporation rates obtained were similar to those with the heterologous material. This was in agreement with the results of Katz et al. (7) who found similar rates of albumin synthesis with $NaH^{14}CO_3$ as a precursor using either rat- or human-heparinized red cells in the perfusion medium, and the observations of John and Miller (8) who reported that the use of heterologous blood did not affect liver functions.

Algal protein hydrolysate containing U-[^{14}c]amino acids, L-U-[^{14}C]leucine, and 9,10-[^{3}H]oleic acid were used as tracers (The Radiochemical Centre, Amersham, Great Britain). The perfusion with labeled amino acids lasted for 3 hr after the addition of the radioactive tracer, whereas the perfusion with labeled oleate-albumin complex was carried out for 2 hr.

Incorporation into Total Perfusate Proteins

The red cells were centrifuged out from samples of perfusate and 1-ml portions of the supernatant fluid were treated with trichloroacetic cid (TCA) at a final concentration of 10%. The precipitate was washed 3 times with 5% TCA and 2 times with ethanol/ether (3:1, v/v). The radioactivity in the proteins was determined after dissolving the precipitate in 1 ml of concentrated formic acid (9) and counting in a liquid scintillation spectrometer, using a toluene-ethanol scintillator. All radioactivities were corrected for quenching and recorded as disintegrations/min (dpm). The incorporation data were expressed as pro mille (‰) of the substrate radioactivity in the total perfusate vol at 0 time, e.g., after equilibration time of 3-5 min from the start of the perfusion.

Separation of Lipoproteins and Albumin from the Perfusate

The lipoproteins were floated from 10- to 15-ml samples of the cell-free perfusate by successive centrifugations in a Model L3-50 Spinco ultracentrifuge as described in detail elsewhere (1). One exception was the addition of dialyzed normal fasting rat serum as a carrier in the final dilution of 1:25. The first fraction was separated by centrifugation at d=1.063 g/ml and regarded as very low density lipoproteins (VLDL) because of the negligible contribution of low density lipoproteins (LDL) by the isolated perfused liver. The radioactivity in the protein and lipid moieties of the isolated lipoproteins, washed once by recentrifugation, was measured after extracting with chloroform/methanol (2:1, v/v) as outlined (1).

Separation and Counting of Proteins and Lipids in the d>1.21 g/ml Fraction

The infranatant fraction of the perfusate remaining at d=1.21 g/ml, was dialyzed against 0.9% NaCl. The lipids were extracted from one portion in chloroform/methanol, washed and counted. The proteins from another portion of the infranatant were precipitated with TCA and the albumin was extracted with a solution of 1% TCA in 95% ethanol (w/v) and counted as described (1).

Radioactivity in Liver Proteins and Lipids

Liver proteins were precipitated at 10% TCA concentration from a 25% liver homogenate in 0.25 M sucrose prepared at the end of perfusion. Label incorporation into the total liver lipids was measured by extracting a 1.0-g sample into chloroform/methanol. The procedures for protein and lipid washing and counting were as described for soluble proteins (1).

Control Experiments

Heparin was included in the liver perfusate as recommended by Jones et al. (10), Haft et al. (11), Faloona et al. (12) and Tracht et al. (6) in their lipoprotein synthesis studies. The amount added (5 units/ml) was generally lower than that used by other investigators but was found helpful in prolonged perfusions required to demonstrate amino acid incorporation into lipoproteins as it prevented an occasional clotting of the newly synthesized fibrinogen within the hepatic circulation and thus improved the flow rate. In several experiments performed without the addition of heparin, the rate of precursor incorporation into the lipoprotein protein moiety was reduced by 1/3 to 1/2 in the livers of both control and nephrotic rats. To assess the extent of lipolysis in the perfusate, the content of FFA was determined by titration. Rises during the 3-hr perfusion with amino acids were on the order of 16% above the low initial value of 0.15-0.26 μmol/ml at most. Had any preferential lipolysis of newly synthesized lipids occurred, one would expect high radioactivity of the FFA bound to albumin in the d>1.21 g/ml fraction, which was not the case. Furthermore, incubation of the perfusate in vitro at the end of perfusion

for an additional hr at 37 C did not result in a decrease of esterified lipid radioactivity by more than 7% when measured in experiments with algal amino acids, leucine or oleate as precursors of the labeled perfusate lipids. These results negate the possibility of an appreciable or sustained lipolysis of the perfusate lipids.

To assess the contamination of lipoprotein components with nonincorporated precursor label, a portion of the medium was removed at 0 time and ultracentrifuged in parallel. The radioactivity retained in the washed chloroform/methanol extracts or in the apolipoprotein fractions was in the range of 1-3% of that in the corresponding fractions removed at the end of perfusion and in each experiment it was subtracted as a blank.

RESULTS

Figure 1 documents the linear increase with time of the incorporation of [^{14}C]amino acids from the algal hydrolysate into the total perfusate proteins by the livers of control, nephrotic and plasmapheretic rats. A significantly increased net incorporation into the total perfusate proteins secreted by the liver of the hypoproteinemic rats may be seen.

Table I shows a significant increase in [^{14}C] content of VLDL and high density lipoprotein (HDL) apolipoproteins and of albumin separated from the liver perfusate of nephrotic rats. Since the liver size of the nephrotic rats is increased, the amino acid incorporation was calculated both per g tissue and per amount of liver corresponding to 100 g body wt of the intact rat. This was done to properly express the capacity of the total liver of the nephrotic or plasmapheretic animal to incorporate lipoprotein precursors in relation to the control animal. As seen in Table I, the mean increase in incorporation of amino acids was most pronounced in the case of VLDL amounting to 3.5 times that in control rats. With HDL, the increase was ca. 2-fold and with albumin it was about 3-fold. The incorporation values in the liver perfusate from plasmapheretic rats, although significantly higher than those of normal rats, were lower when compared with nephrotic rats.

Table II shows that no increase in the incorporation of the [^{14}C]amino acids was detected in the lipoprotein-borne lipids secreted into the perfusate by the liver of nephrotic or plasmapheretic rats. In the nephrotic rats, even a lower-than-normal incorporation into the VLDL was observed. There was a slight increase in the amount of [^{14}C] label in the fraction sedimenting at d=1.21 g/ml. However, as far

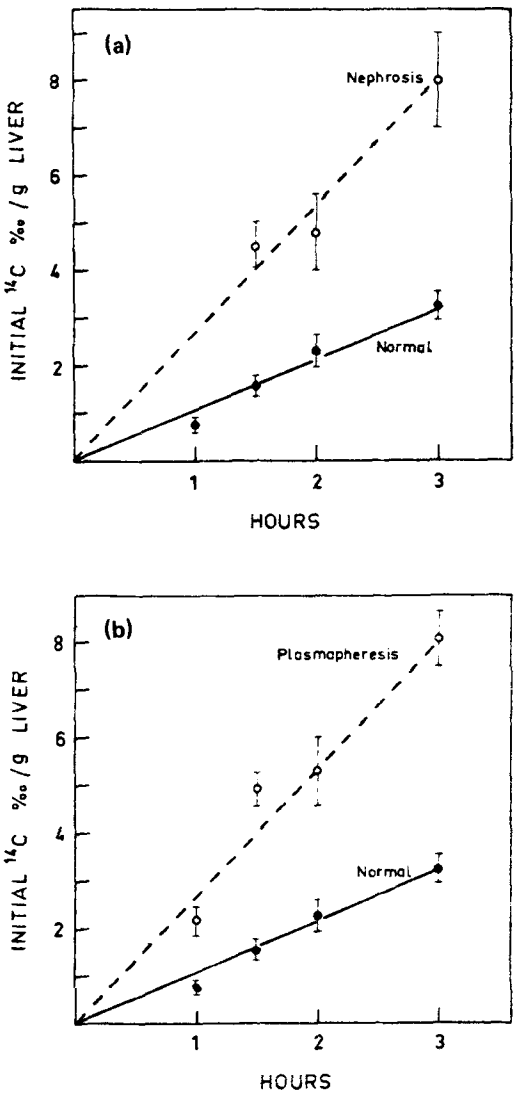

FIG. 1. Time course of amino acid incorporation into total liver perfusate proteins in nephrotic (a) and plasmapheretic (b) rats. Vertical bars represent mean ± SE of 8-10 determinations in each group. The values given are ‰ of initial radioactivity of the amino acid hydrolysate in the total medium which amounted to 5 μc.

as the sum of incorporation into the lipids of all fractions is concerned, there was no significant change. To ascertain that lipid synthesis was not increased because of deficiency of an appropriate amino acid precursor in the algal-derived mixture, additional perfusions were carried out using U-[^{14}C]leucine as a single amino acid tracer which was added in a high concentration of 2 μmol/ml perfusate, with other nonlabeled amino acids of the mixture

remaining at physiological concentration (5). The results in Table III show a 5-fold increase in the incorporation into total perfusate proteins (per 100 g body wt) secreted by the liver of nephrotic rats. This increase was greater than that obtained with the [^{14}C]-labeled algal amino acid mixture. However, even with leucine at high concentration, there was no statistically significant increase in the incorporation into total perfusate lipids when calculated per g liver wt although a 2-fold, but barely significant increase was obtained when expressed per 100 g body wt.

Determinations also were made of the labeling of liver tissue proteins and lipids during the perfusion with leucine. They showed small increases in incorporation of leucine radioactivity into hepatic proteins or lipids in nephrosis which became significant only when calculated per 100 g body wt (Table III).

Since marked hyperlipoproteinemia was evident in the serum of the nephrotic rats, the role of FFA as a source of lipoprotein-borne lipids was then investigated by perfusing the liver with [^{3}H]-labeled, albumin-bound oleate as a source of the preformed lipid (Table IV).

TABLE I

Incorporation of ^{14}C-Amino Acids into Rat Liver Perfusate Apolipoproteins and Albumin

Fraction	Normal (14)	Nephrosis (14)	Plasmapheresis (10)
		‰ Initial radioactivity/3 hr	
VLDL (d<1.063)			
per g tissue	0.116 ± 0.012	0.284 ± 0.020[a]	0.205 ± 0.022[a]
per 100 g body wt	0.458 ± 0.049	1.60 ± 0.12[a]	0.775 ± 0.084[a]
HDL (1.063<d<1.21)			
per g tissue	0.065 ± 0.006	0.096 ± 0.009[a]	0.089 ± 0.009[b]
per 100 g body wt	0.259 ± 0.023	0.538 ± 0.047[a]	0.347 ± 0.035[b]
Albumin			
per g tissue	3.08 ± 0.40	6.62 ± 0.71[a]	5.72 ± 0.68[a]
per 100 g body wt	12.2 ± 1.7	37.1 ± 4.3[a]	22.0 ± 2.6[a]

Values are means ± SE for groups of rats indicated in parentheses. The initial medium contained 5 μc of amino acid radioactivity. The mean ratio liver/body weight was 4.0 ± 0.1, 5.6 ± 0.3 and 3.8 ± 0.2 in normal, nephrotic and plasmapheretic rats, respectively.

[a]Differences from control values significant at $P < 0.01$ at least.

[b]Differences from control values significant at $P < 0.05$ at least.

TABLE II

Amino Acid Label in the Lipid Moiety of Rat Liver Perfusate Lipoproteins

Fraction	Normal (12)	Nephrosis (10)	Plasmapheresis (8)
		‰ Initial radioactivity/3 hr	
VLDL (d<1.063)			
per g tissue	0.294 ± 0.035	0.134 ± 0.020[a]	0.229 ± 0.023
per 100 g body wt	1.18 ± 0.14	0.754 ± 0.110[a]	0.877 ± 0.089
HDL (1.063<d<1.21)			
per g tissue	0.034 ± 0.002	0.028 ± 0.006	0.030 ± 0.004
per 100 g body wt	0.136 ± 0.010	0.156 ± 0.034	0.114 ± 0.015
Lipids (d>1.21)			
per g tissue	0.176 ± 0.018	0.184 ± 0.017	0.196 ± 0.014
per 100 g body wt	0.700 ± 0.072	1.03 ± 0.11[b]	0.745 ± 0.054

Values are means ± SE for groups of rats indicated in parentheses. For calculation of incorporation per 100 g body wt, see the respective liver wt data in Table I.

[a]Significant decrease compared to control value at $P < 0.01$.

[b]Significant increase compared to control value at $P < 0.02$.

TABLE III

Incorporation of Leucine into Liver Perfusate and Liver Tissue Proteins and Lipids

	Control	Nephrosis
	Total perfusate proteins	
nmol/2 hr • g liver tissue	59 ± 6 (18)	204 ± 25[a] (10)
nmol/2 hr • 100 g body wt	234 b 23 (18)	1140 ± 136[a] (10)
	Total liver proteins	
nmol/2 hr • g liver tissue	38 ± 5 (10)	45 ± 6 (8)
nmol/2 hr • 100 g body wt	153 ± 19 (10)	254 ± 28[a] (8)
	Total perfusate lipids	
nmol/2 hr • g liver tissue	16 ± 4 (18)	26 ± 6 (10)
nmol/2 hr • 100 g body wt	65 ± 15 (18)	144 ± 34[b] (10)
	Total liver lipids	
nmol/2 hr • g liver tissue	61 ± 9 (10)	84 ± 17 (8)
nmol/2 hr • 100 g body wt	242 ± 37 (10)	476 ± 94[b] (8)

Values are means ± SE for groups of rats indicated in parentheses. The perfusion medium (100 ml) contained 5 μCi and 200 μmol U-[^{14}C]leucine and a mixture of nonlabeled amino acids at physiological concentration (see Experimental). For calculation of leucine incorporation the initial specific activity was taken since the content of leucine in either normal or nephrotic liver (1) was negligible with regard to the amount of leucine in perfusate. For calculation of incorporation per 100 g body wt, see the respective liver wt data in Table I.

[a]Difference from control value significant at $P < 0.01$ at least.

[b]Difference from control value significant at $P < 0.05$.

A 3- to 4-fold increase was noted in the incorporation of oleate label into the VLDL and HDL lipids secreted into the perfusate. No significant rise was noted in the incorporation of oleate label into the endogenous liver lipids, pointing out that the nephrosis-induced increment in oleate incorporation was directed into the lipoprotein-borne lipids rather than into the cellular liver lipids.

DISCUSSION

Supplementing our in vivo work with nephrotic and plasmapheretic rats, these experiments confirm the higher-than-normal rate of production of apolipoproteins and albumin by isolated, perfused liver. The observations of increased hepatic rate of amino acid incorporation extend the evidence on enhanced plasma protein synthesis in nephrosis, obtained previously with the aid of immunological and chemical measurements of lipoproteins released from the liver (13,14) and the augmented hepatic albumin synthesis using $NaH^{14}CO_3$ as a marker (7). In particular, in the isolated perfused liver of nephrotic rats an increase in amino acid flow into the apoproteins of both VLDL and HDL can be demonstrated. In our previous findings in intact rats (1), we noted a 4.5-fold increase in VLDL label and a 2.5-fold increase in HDL label 1 hr after leucine injection. In this study, the respective increases were 3.5- and 2-fold 3 hr after the perfusion with an amino acid mixture.

Amino acids are mobilized in nephrosis from peripheral tissues at an increased rate and actively metabolized in the liver. We have previously demonstrated in nephrosis a rise in muscle proteolytic activity (15), a higher rate of plasma amino acid transport (16) and greater than normal hepatic breakdown of amino acids as a result of increased activity of several amino acid-metabolizing enzymes in the liver (17). Our present results make it apparent that, despite their greater availability, amino acids do not significantly contribute to the increased elaboration of lipids in nephrosis or plasmapheresis and may represent poor lipoprotein lipid precursors in general. No increment was observed in the lipoprotein-lipid label secreted by the liver from nephrotic or plasmapheretic rats with an amino acid mixture at physiological concentration. With leucine as a donor of labeled acetyl-CoA carbons, even at >10 times higher than physiological concentration, only small and barely significant rises in lipid label were obtained.

Comparing the data of Tables I and II, we may note that with control rat livers more amino acid radioactivity was incorporated into the lipid than protein moiety of the VLDL (1.18 ± 0.14 vs 0.458 ± 0.049‰ of the perfusate

TABLE IV

Incorporation of Oleate into Perfusate Lipoprotein Lipids and Liver Tissue Lipids

Fraction	Control	Nephrosis
	Percent of initial radioactivity/2 hr	
VLDL (d<1.063)		
per g tissue	0.48 ± 0.05	1.43 ± 0.18[a]
per 100 g body wt	1.90 ± 0.19	7.86 ± 1.02[a]
HDL (1.063<d<1.21)		
per g tissue	0.11 ± 0.03	0.31 ± 0.04[a]
per 100 g body wt	0.45 ± 0.12	1.72 ± 0.21[a]
Total Liver Lipids		
per g tissue	3.27 ± 0.39	3.11 ± 0.41
per 100 g body wt	13.10 ± 1.60	17.38 ± 2.26

Values are means ± SE for groups of 8 rats. The perfusion medium (100 ml) initially contained 5 μCi and 200 μmol of 9,10-[^{3}H]potassium oleate complexed to 5% FFA-poor bovine albumin. The VLDL were separated by ultracentrifugation and extracted as described for amino acid perfusate. The HDL were washed once by recentrifugation in the presence of fresh FFA-poor 5% albumin solution. For calculation of incorporation per 100 g body wt, see the respective liver wt data in Table I.

[a]Difference from control value significant at $P < 0.005$.

label/3 hr/100 g body wt). This may be related to the fact that the total lipid/protein content ratio in the VLDL exceeds 10. However, in the VLDL produced by the liver of nephrotic rats, the lipid/protein incorporation ratio was much lower (0.765 ± 0.110 vs. 1.60 ± 0.12‰ of the perfusate label per 3 hr per 100 g body wt). It is unlikely that this change in amino acid channeling resulted from a decreased lipid content of the VLDL in nephrosis. Although the actual lipid/protein content ratios in the secreted VLDL have not been determined because of the addition of carrier lipoproteins, the plasma VLDL of the same control and nephrotic rats had triglyceride/protein ratios of 7.8 ± 0.8 and 6.6 ± 0.4, respectively (unpublished results). The size of liver triglyceride pool in our aminonucleoside nephrotic rats is also similar to that of control rats (17). Thus, the relative decrease in incorporation into the lipid moiety of the lipoproteins in favor of the increase in incorporation into apolipoproteins in nephrosis (and to a lesser extent also in plasmapheresis) apparently results from a preferential amino acid flow into peptide synthesis. Our previous finding that gluconeogenesis capacity is reduced in nephrosis (18) fits into this pattern, since it indicates that amino acids are spared from metabolism along pathways of less immediate importance than plasma protein synthesis.

The ratio of leucine label appearing in perfusate lipids to that remaining in the endogenous liver lipids was ca. 0.3 in the control and nephrotic rat livers alike (Table II). The perfusate/liver ratio of leucine label incorporated into proteins was much higher: ca. 1.5 in the control rat liver rising to 4.5 in nephrosis. While the conclusion is that nephrosis did not prommote, or even cause a decrease in the channeling of amino acids into lipids, as illustrated in the VLDL label ratio, it may be added that leucine and other amino acids may not represent good precursors for secretable lipids in general, as seen from the retention of most of leucine-derived lipid in liver cells, in contrast to the release of most of leucine-labeled proteins.

Preformed FFA participate to an increased extent in the elaboration of lipoprotein-borne lipids in nephrosis. This was indicated from the time-course of changes in the circulating metabolites during the development of aminonucleoside nephrosis (17): the joint onset of hypoproteinemia and hyperlipoproteinemia was temporally correlated with marked mobilization of FFA from the peripheral fat depots. Increased lipolytic activity and FFA release was also demonstrated in adipose tissue of aminonucleoside-nephrotic rats (19) and in isolated fat cells of antikidney serum nephrotic rats (20). As reviewed by Edwards (21), support for the increased contribution of FFA to the synthesis of lipoprotein-borne lipids in nephrosis was also obtained from studies of in vivo injected FFA and their reappearance in circulating lipids (22,23), and recently from studies demonstrating rapid hepatic esterification of preformed FFA in chronic nephrotic rats (24).

The results presented in Table IV directly

demonstrate the enhanced uptake of FFA by the isolated liver of nephrotic rats and their increased recirculation as lipoprotein-borne esterified lipids, underscoring the importance of the supply of preformed extrahepatic fatty acids for the assembly of lipoproteins in nephrosis. It may be added that the initial perfusate oleate/albumin molar ratio in these experiments was ca. 2.6 which is higher than that in the plasma of normal rats but commonly encountered in the plasma of nephrotic rats (1,17) or human subjects (25). This occurs as a result of decreased albumin and increased FFA levels. At an oleate/albumin molar ratio close to 1 prevailing in the plasma of control rats, the removal of oleate by the nephrotic rat liver was too rapid to sustain a linear rate of disappearance during the 2 hr of perfusion.

It may be concluded that hepatic overproduction of apolipoproteins occurs within the frame of a general compensatory response to hypoproteinemia, elicited whether protein is spilled out to the urine in nephrosis or is directly removed by plasmapheresis. The enhanced elaboration of lipids is probably a sequel to the excessive availability of apolipoproteins, into the syntheis of which amino acids are channeled in preference. The requirement for the lipid complement must be met, then, by increased new fatty acid synthesis as demonstrated with substrates like acetate (13,26), glucose (27) or citrate (1), or, as shown here, by the enhanced hepatic use of preformed FFA. When amino acids and FFA are offered, their export into the circulation as protein and lipid moieties of the lipoproteins, respectively, is favored over their incorporation into hepatic cellular proteins or lipids, whereas the amino acid-derived lipids are mostly retained in the liver.

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Effect of Feeding Protected Lipids on Fatty Acid Synthesis in Ovine Tissues

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ABSTRACT

The effects of including protected lipid supplements in the sheep diet have been studied by measuring the incorporation of [1-^{14}C]acetate into tissue fatty acids in vivo and in vitro. Supplementing the diet with protected lipid significantly ($P < 0.05$) depressed lipogenesis in adipose tissue both in vivo and in vitro. However, when protected lipids of different fatty acid composition were given to lambs, the protected safflower oil supplement containing high levels of linoleic acid was the only treatment to cause a significant ($P < 0.05$) depression in fatty acid synthesis in adipose tissue, the major site of lipogenesis in the sheep. Larger adipose cells in the lipid-supplemented sheep indicate that these sheep were fatter than those receiving the basal diet. Therefore, supplemented wethers deposited more fat than sheep receiving the basal diet and this fat was derived from the supplement rather than from de novo synthesis.

INTRODUCTION

Under conventional feeding regimes, ruminants are unable to tolerate high levels of lipid in the diet. However, the intake of lipid can now be increased by feeding it as emulsified oil droplets coated with a layer of formaldehyde-treated protein (1-3). Besides allowing a modification of the fatty acid composition of ruminant meats and dairy products (3-5), which has important medical implications, the feeding of protected lipid offers a unique opportunity to study various aspects of lipid metabolism in sheep and cattle. Unless offered in a protected form, the possible effects of dietary polyunsaturated fatty acids upon lipid metabolism of ruminant tissues are not observed because of biohydrogenation of these acids by rumen microflora (6-8).

Diets which are high in lipid are known to depress the rate of lipogenesis in mammalian tissues (9-15). Evidence obtained with the mouse (16-18) and the rat (19) suggests that diets containing high levels of linoleate have a greater inhibitory effect on the rate of lipogenesis than diets containing equivalent levels of saturated or monounsaturated fatty acids. However, other studies with the rat (9,20,21) and the pig (13) suggest that the fatty acid composition of the dietary lipid is not important for the depression of fatty acid synthesis. The primary role of linoleic acid in exerting an extra-inhibitory effect on the rate of lipogenesis in mammalian tissues of some species is unknown.

This study was designed to investigate the effect of feeding protected lipids of different fatty acid composition on the rates of in vitro and in vivo lipogenesis in sheep.

MATERIALS AND METHODS

Animals and Diets

Eight Merino wethers (castrated male sheep), 5 years old, were kept indoors in individual pens. Four were fed an experimental diet consisting of chopped alfalfa (700 g) and crushed oats (250 g) daily, supplemented with 500 g formaldehyde-treated sunflower oil-casein (8.71 kcal/g dry wt) (2) for 12 wk. The remaining 4 animals were fed a basal diet of chopped alfalfa (400 g) and crushed oats (1100 g) daily.

In a second experiment, 9 crossbred Merino lambs (16-wk-old) were fed 210 g daily of a supplement of formaldehyde-treated casein (1 part) containing either tallow (2.4% linoleic acid [18:2]), palm oil (9.1% 18:2), or safflower oil (59.8% 18:2, 2 parts) together with chopped alfalfa (350 g) and crushed oats (140 g). Three lambs were fed a basal diet of chopped alfalfa (400 g) and crushed oats (400 g) for the 6-wk experiment run.

Tissue Samples

All tissues were excised from the animals immediately after death. Subcutaneous (SC) adipose tissue samples were taken from above the last rib, and perirenal adipose tissue was sampled adjacent to the left kidney. Omental adipose tissue was taken from the greater omentum. The cellularity of intramuscular adipose tissue was determined on tissue dissected from a section of longissimus dorsi muscle taken at the last rib. A length of small

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intestine was removed 50 cm from the abomasum and the mucosa isolated by scraping the intestinal wall with a glass plate.

Incubations (in vitro)

Thin slices (< 1.0 mm) of adipose tissue and liver were prepared with a razor blade from the freshly excised tissues. Each tissue slice (124-200 mg) or section of intestinal mucosa was weighed and placed in a 25-ml Erlenmeyer flask containing 10 μmol [1-^{14}C] acetate and 10 μmol glucose in 3 ml of Ca^{2+} free Krebs-Ringer bicarbonate buffer at pH 7.4. The flasks with mucosa and adipose tissue also contained 0.3 units of insulin (Eli Lilly-Aust. Co., Sydney, Australia). Each flask contained a 10 x 34 mm glass vial containing 0.1 ml 25% KOH and a strip of filter paper to trap $^{14}CO_2$. Each flask was flushed with oxygen, sealed with a rubber serum cap and incubated at 37 C in a reciprocating water bath for 2 hr. The reaction was terminated by the injection of 0.25 ml of 1 M H_2SO_4 through the serum cap and into the incubation medium. Methodology for the extraction of [^{14}C] crude lipid and the estimation of $^{14}CO_2$ was described previously (22).

The radioactivity in an aliquot of the [^{14}C] crude lipid was measured with a liquid scintillation spectrometer (Packard Instrument Pty. Ltd.) and the remainder of the [^{14}C] crude lipid was transferred in $CHCl_3$ to a 1 x 6 cm test tube for esterification. This was achieved through drop-by-drop addition of sufficient 1.25 M sodium methoxide (containing 0.01% phenolphthalein) to keep the reaction mixture alkaline (23). After 30 min at room temperature, the reaction mixture was neutralized with 10% hydrochloric acid in methanol. The $CHCl_3$ layer now contained [^{14}C] crude lipid in the form of fatty acid methyl esters and cholesterol. These 2 lipid classes were separated using fiber glass sheets impregnated with silica gel (Gelman Instrument Co., Ann Arbor, MI). The sheets were cut into 1.5 x 115 cm strips and activated at 110 C for 10 min in preparation for the chromatographic separation of cholesterol and methyl esters. About 100 μg of the [^{14}C] lipid mixture was spotted onto the chromatographic strip in 5-μl aliquots, together with 50 μl of a standard mixture. Development was done using hexane/diethyl ether/acetic acid (98:1:0.15) and the spots were detected by spraying with 0.2% dichlorofluorescein in ethanol. The spots were cut out, placed in a counting vial containing 10 ml of Brydet scintillation solution (24) and the radioactivity determined. The proportion of radioactivity in the methyl ester spot multiplied by the total [^{14}C] lipid synthesized was used to measure the in vitro incorporation of [1-^{14}C] acetate into fatty acids. Since the same animals were used for both in vitro and in vivo experiments, corrections were made for the initial radioactivity in the tissue slices.

Lipogenesis in vivo

Radioactive sodium [1-^{14}C] acetate (340 μCi) in 10 ml of sterile saline was injected intravenously into each sheep 30 min before slaughter. The injection of radioactivity was followed by 2 further injections of saline (5 ml each) to rinse the syringe and needle. After exsanguination, samples (1-2 g) of adipose tissue, liver, duodenum, heart and skeletal muscle were accurately weighed and saponified by heating at 80 C for 2 hr with 10 ml of 5 N NaOH/95% ethanol (1:1) (25). The saponifiable tissue lipids (fatty acids) were extracted with petroleum ether and the incorporation of radioactivity into fatty acids was measured with a liquid scintillation spectrometer.

Enzyme Assays

The preparation of the 48,500 x g supernatant used in the enzyme assays and the assays for glucose-6-phosphate dehydrogenase (G6-PDH) (E.C. 1.1.1.49) and 6-phosphogluconate (6-PGDH) (E.C. 1.1.1.44) have been described elsewhere (26). The activities of adipose tissue enzymes and the rates of in vitro lipogenesis were compared on a cellular basis since an equal number of adipose cells has been shown to be the preferred reference unit for the comparison of enzyme activities between adipose tissues containing cells of different sizes (26). Adipose tissue cellularity was determined on osmium tetroxide-fixed adipose cells using a modification of the Coulter counter technique described by Hirsch and Gallian (27). Tabular data are expressed as means ± standard error of the mean (SEM).

RESULTS

Lipogenesis

The addition of safflower oil protected with formaldehyde-treated casein to the diet of 5-year-old wethers resulted in a depressed rate of in vitro lipogenesis in the SC ($P < 0.05$) adipose tissue from these sheep when compared to wethers fed the control diet (Table I). Measurement of in vivo lipogenesis on the same animals also indicated depressed lipogenesis ($P < 0.05$) in the 4 adipose tissues studied (Table II) from the sheep given the protected lipid supplement. When the in vivo data (Table II) was plotted on the basis of an equal number of adipose cells (Fig. 1), the difference between

TABLE I

In vitro Lipogenesis in Ovine Tissue from Sheep Fed Protected Dietary Lipid[a]

Adipose tissue	nmol [1-^{14}C] acetate to fatty acids/2 hr/10^6 adipose cells	
	Basal diet	Supplement diet
Subcutaneous	4646[b] ± 268	1998[c] ± 332
Perirenal	1692 ± 341	1380 ± 294

[a]Data from sheep in experiment 1; 4 sheep per treatment.

[b,c]Means on the same row with different superscripts are significantly different ($P < 0.05$).

TABLE II

In vivo Incorporation of ^{14}C-Acetate into Fatty Acids in Tissues of Wethers[a]

Tissue	dpm/g tissue/μC/kg live weight	
	Basal diet	Supplement diet
	Fatty acids	
Shoulder subcutaneous adipose tissue	2036[b] ± 617	720[c] ± 235
Rib subcutaneous adipose tissue	2394[b] ± 279	790[c] ± 217
Omental adipose tissue	1360[b] ± 212	370[c] ± 128
Perirenal adipose tissue	1908[b] ± 334	228[c] ± 36
Liver	697[b] ± 186	326[c] ± 51
Duodenum	240 ± 6	307 ± 44
Heart muscle	127 ± 24	132 ± 11
Skeletal muscle	353 ± 135	245 ± 11

[a]Data from sheep in experiment 1; 4 sheep per treatment.

[b,c]Means on the same row with different superscripts are significantly different ($P < 0.05$).

treatment groups was diminished because of the presence of larger adipose cells in the tissues from the sheep receiving supplement. In both the in vitro and in vivo studies, the perirenal adipose tissue from the sheep receiving supplement was less active in lipogenesis than the SC adipose tissue. In the SC and perirenal adipose tissues, the activities of 6-PGDH and G6PDH were lower in those animals receiving the protected lipid supplement (Table III).

Under in vivo conditions the incorporation of [^{14}C] acetate into fatty acids was lower ($P < 0.05$) in the livers from the sheep on supplement, whereas the incorporation was similar in both groups for heart and skeletal muscle. On the other hand, those sheep receiving supplement converted more [^{14}C] acetate to fatty acid in the duodenum than the sheep on the basal diet (Table II). Considering the relative weights of skeletal muscle and adipose tissue, skeletal muscle apparently is a significant site for the incorporation of [1-^{14}C] acetate into fatty acids.

When fats of differing fatty acid composition were protected from ruminal hydrogenation, the protected safflower oil, an oil containing ca. 60% linoleic acid, was the only protected lipid supplement to reduce ($P < 0.05$) lipogenesis in SC and perirenal adipose tissue of lambs used in experiment 2 (Table IV). In the lambs on the 3 diets containing protected lipid, hepatic lipogenesis was depressed ($P < 0.05$) in each case when compared to the lambs on the control diet. No significant differences were observed in the rates of lipogenesis in the intestinal mucosa from the lambs in the 4 treatment groups (Table IV). The major portion of the total lipid synthesized in the 3 tissues listed in Table IV was recovered as fatty acids. However, in the intestinal mucosa, cholesterol synthesis makes a significant contribution to the total lipid synthesized (25).

Adipose Tissue Cellularity

The average volume of the adipose cells from the tissues of 5-year-old wethers is shown in Table V. The extramuscular adipose tissues, i.e., subcutaneous, perirenal and omental from the sheep fed protected safflower oil had larger (P

< 0.01) adipose cells than those found in the corresponding adipose tissues from the sheep on the basal diet. No difference was observed in the average volume of the intramuscular adipose cells from the longissimus dorsi muscle of the wethers in the 2 groups. A similar result was obtained in the SC adipose tissue of lambs in experiment 2 (Table IV), i.e., the lambs fed protected safflower oil had larger adipose cells than those sheep receiving no lipid supplementation. Those animals receiving protected tallow and protected palm oil had SC adipose cells which were intermediate in size to those found in the lambs fed either a basal or a protected safflower oil diet (Table IV). A similar trend was apparent for the perirenal adipose tissue from the same sheep fed the protected lipid supplements (Table V).

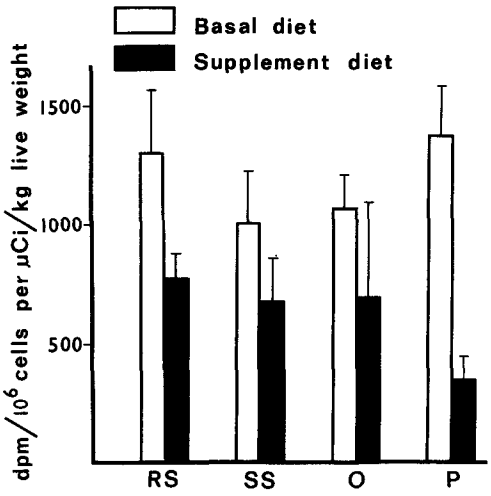

FIG. 1. In vivo incorporation of [^{14}C]acetate into fatty acids in adipose tissues of wethers. RS = Rib subcutaneous adipose tissue; SS = shoulder subcutaneous adipose tissue; O = omental adipose tissue; P = perirenal adipose tissue.

DISCUSSION

This study indicates that dietary lipid, when fed to wethers as emulsified oil droplets coated with a layer of formaldehyde-treated protein, results in a depression in fatty acid synthesis (Tables I and II). However, when protected lipids of different fatty acid composition were fed to lambs, only the protected safflower oil supplement, containing high levels of linoleate, caused a significant ($P < 0.05$) inhibition in fatty acid synthesis (Table IV). Nestel et al. (25) also found a reduced in vitro incorporation of [1-^{14}C]acetate to triglycerides, phospholipids and sterols in ovine hepatic tissue.

In mammalian tissues, the feeding of diets containing a high proportion of lipid results in a depression of lipogenesis (9-15). An early report (28) suggests that inhibition of lipogenesis by feeding of fat may be localized at the step involving the carboxylation of acetyl CoA. The mechanisms by which dietary lipid inhibits fatty acid synthesis are unclear. Elevated levels of circulating free fatty acids have been observed under conditions of reduced fatty acid synthesis as a result of including dietary lipid into the pig diet (12). Free fatty acids or their coenzyme A derivatives have been reported (29,30) to inhibit acetyl CoA carboxylase, which has been suggested as a rate-limiting step in fatty acid synthesis (31). Fatty acyl-CoA may inhibit fatty acid synthesis by directly inhibiting acetyl-CoA carboxylase or by inhibiting the mitochondrial carrier, thereby reducing the activation of acetyl-CoA carboxylase caused by citrate (32). However, Dorsey and Porter (33) have questioned the physiological significance of the inhibitory effect of these compounds, since they affect a large variety of enzyme systems.

Decreased fatty acid synthesis in the animals supplemented with protected lipid was also indicated by a decreased activity of G6PDH and 6-PGDH (Table III), 2 important NADPH-

TABLE III

Enzyme Activity in Ovine Adipose Tissue[a,b]

	nmol Substrate used/min/10^6 adipose cells			
	Glucose-6-phosphate dehydrogenase[a]		6-phosphogluconate dehydrogenase[a]	
Adipose tissue	Basal diet	Supplement diet	Basal diet	Supplement diet
Subcutaneous	139 ± 8	68 ± 3	577 ± 36	257 ± 35
Perirenal	127 ± 3	48 ± 5	521 ± 34	170 ± 29

[a]Data from sheep in experiment 1; 4 sheep per treatment.

[b]For each enzyme within each tissue, the basal diet was significantly higher ($P < 0.05$) than the supplement diet.

TABLE IV

The Effect of Fatty Acid Composition of a Protected Lipid Supplement on in vitro Lipid Metabolism[a]

Parameter	Basal diet	Protected lipid supplement		
		Tallow	Palm	Safflower
Subcutaneous (SC) adipose cell volume (nℓ)	0.44[e] ± 0.09	0.71[e] ± 0.06	0.57[e] ± 0.03	0.95[d] ± 0.07
Perirenal adipose cell volume (nℓ)	0.58[e] ± 0.10	0.78[d] ± 0.04	0.68[e] ± 0.04	0.95[d] ± 0.08
Nanomoles of [1-^{14}C]acetate converted to total lipid/2 hr in:				
SC adipose tissue[b]	6337[e] ± 1267	3696[e] ± 726	4329[e] ± 524	1579[d] ± 234
perirenal adipose tissue[b]	1876[e] ± 362	1744[e] ± 262	2406[e] ± 423	994[d] ± 123
liver[c]	12.1[d] ± 1.5	4.4[e] ± 1.1	4.0[e] ± 1.4	4.6[e] ± 0.9
intestinal mucosa[c]	11.1 ± 1.0	10.2 ± 1.7	11.8 ± 1.6	10.9 ± 1.2
Percent of de novo synthesized lipid as fatty acid in:				
SCA adipose tissue	98	99	98	98
liver	88	98	99	98
intestinal mucosa	81	75	71	76

[a]Data from lambs in experiment 2; 3 lambs per treatment.

[b]Lipogenesis rate expressed/10^6 adipose cells.

[c]Lipogenesis rate expressed/mg soluble protein.

[d,e]Means on the same row with different superscripts are significantly different ($P < 0.05$).

TABLE V

Cellularity of Ovine Adipose Tissue[a]

Adipose tissue	Adipose cell volume (nℓ)	
	Basal diet	Supplement diet
Subcutaneous	0.52[b] ± 0.09	1.15[c] ± 0.15
Perirenal	0.73[b] ± 0.17	1.53[c] ± 0.11
Omental	0.80[b] ± 0.13	1.46[c] ± 0.13
Intramuscular	0.26 ± 0.10	0.32 ± 0.19

[a]Data from sheep in experiment 1; 4 sheep per treatment.

[b,c]Means on the same row with different superscripts are significantly different ($P < 0.01$).

generating enzymes which reflect the status of fatty acid synthesis in ruminant adipose tissue (34).

In the mouse (16-18) and the rat (19), the degree of inhibition of lipogenesis by dietary lipid has been related to the linoleate content of the dietary lipid. In contrast, other studies with rats (9,20,21) report that fatty acid composition does not affect fatty acid synthesis. By supplementing the diet of cows' milk with safflower oil (5 ml/lamb/day), Vernon (14) reported significantly lower rates of lipogenesis in adipose tissue slices from 11-day-old lambs. Allee et al. (13) have demonstrated that a high linoleate content of dietary lipid is not a prerequisite for the inhibition of fatty acid synthesis in pig adipose tissue since all fats (corn oil, tallow, lard and coconut oil) were equally effective in depressing lipogenesis. The primary role of linoleate in exerting an extra-inhibitory effect on the rate of fatty acid synthesis in tissues of many mammalian species is unknown. Adipose tissues, the major site for lipogenesis in ruminant animals (22), are not normally exposed to high levels of circulating linoleate, since unsaturated fatty acids undergo extensive microbial hydrogenation in the rumen (6-8). However, protection of unsaturated fatty acids (e.g., linoleate) from hydrogenation in the rumen enables these fatty acids to be transported unmodified to the adipose tissues for storage. Therefore, this exposes the enzymes involved in fatty acid synthesis to unsaturated fatty acids such as linoleate, which under normal feeding conditions would be present only al low levels. Feeding lambs protected dietary fats (palm oil, tallow) which are low in linoleate caused no significant alteration in fatty acid synthesis (Table IV). Although the mechanisms are unknown, the extra-inhibiting effect of dietary protected linoleate on lipogenesis in ruminant animals is probably at the level of carboxylation of acetyl CoA.

Although fatty acid synthesis was reduced in those sheep supplemented with protected lipid, the volume of their adipose cells was larger than in sheep not receiving the supplement (Tables IV and V). In cattle (35), sheep (36) and pigs (37,38), adiposity in the adult animal has been attributed solely to adipose cell size rather than adipose cell number. On this basis, the supplemented wethers deposited more fat than those receiving the basal diet and this fat was derived from the supplement rather than from de novo synthesis. Hood and Thornton (5) have shown that up to 45% of the lipid in an adipose tissue depot can be derived from a formaldehyde-treated sunflower seed supplement.

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Comparative Studies on Composition of Cardiac Phospholipids in Rats Fed Different Vegetable Oils[1]

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ABSTRACT

Male Sprague-Dawley rats were fed diets for 1 or 16 weeks, containing 20% by weight vegetable oils differing widely in their oleic, linoleic and linolenic acid content. No significant changes were observed in the level of the cardiac lipid classes. The fatty acid composition of the 2 major phospholipids, phosphatidylcholine and phosphatidylethanolamine, showed a remarkable similarity between diets in the concentration of total saturated, C22 polyunsaturated and arachidonic acids. Monounsaturated acids were incorporated depending on their dietary concentration, but the increases were moderate. Dietary linolenic acid rapidly substituted C22 polyunsaturated fatty acids of the linoleic acid family (n-6) with those from the linolenic acid family (n-3). The results suggest that dietary linolenic acid of less than 15% does not inhibit the conversion of linoleic to arachidonic acid but the subsequent conversion of arachidonic acid to the C22 polyunsaturates was greatly reduced. Significant amounts of dietary monounsaturated fatty acids were incorporated into cardiac cardiolipin accompanied by increases in polyunsaturated fatty acids, apparently to maintain an average of 2 double bonds/molecule. The cardiac sphingomyelins also accumulated monounsaturated fatty acids depending on the dietary concentration. It is quite evident from the results of this study that the incorporation of oleic acid and the substitution of linolenic for linoleic acid-derived C22 polyunsaturated fatty acids into cardiac phospholipids was related to the dietary concentration of these fatty acids and was not peculiar to any specific oil. Even though it is impossible to estimate the effect of such changes in cardiac phospholipids on membrane structure and function, results are discussed which suggest that the resultant membrane in the Sprague-Dawley male rat is more fragile, leading to greater cellular breakdown and focal necrosis.

INTRODUCTION

There is extensive evidence to indicate that myocardial lesions in male albino rats fed diets rich in fat or vegetable oil do not result from cardiopathogenic compounds in these fats and oils (1-4) but are related to several dietary fatty acids (5). Linolenic (18:3n-3) (5-8), oleic (18:1n-9) (7,8) and erucic (22:1n-9) acids (6,9) at high levels have been implicated in the etiology of cardiac necrosis, whereas saturated fatty acids (5,10) and linoleic (18:2n-6) acid (7,8) apparently are related to a lower incidence and severity of myocardial lesions in albino male rats.

Dietary fatty acids are known to influence the fatty acid composition of tissue lipids (11). Of several organs tested in the rat, the heart was shown to be most responsive to changes in long-chain polyunsaturated fatty acids (PUFA) when the rats were fed either 18:2n-6 or 18:3n-3 (12). Since these long-chain PUFA are found mainly in phospholipids which are membrane constituents (13), changes in the PUFA may have important consequences in membrane properties and function. These changes, in turn, may be related to the myocardial muscle damage seen in male rats.

In this study, different vegetable oils were chosen to provide a range of dietary fatty acids (i.e., 18:1, 18:2n-6 and 18:3n-3) similar to those found in low erucic acid rapeseed (LEAR) oils, in order to investigate whether the cardiac phospholipid changes in rats fed LEAR oils are peculiar to LEAR oils or simply reflect the dietary fatty acids irrespective of source. Previous studies failed to include control oils that contained similar levels of 18:1 (14), 18:2n-6 (15) and 18:3n-3 (14-19) found in LEAR oils and therefore could not adequately compare the effect of all these dietary fatty acids. In addition, the effects of dietary oils within the first week on the fatty acid composition of the major cardiac phospholipids in the rat were investigated.

MATERIALS AND METHODS

Animals and Diets

Weanling male Sprague-Dawley rats, 3 weeks of age, were supplied by Bio-Breeding, Ottawa, Ontario, and weighed between 40 and 50 g. The rats were distributed randomly to 5 dietary treatments, 5 rats/treatment, and fed ad libitum the test diets for 16 weeks. An additional 10 rats, 5/diet, were fed a diet containing corn oil or LEAR (cv. Zephyr) for 1 week. Five rats were killed immediately after weanling. Water

[1] Contribution No. 914 from the Animal Research Institute.

was available at all times. The semisynthetic diets, described previously (20), contained 20% by weight one of the following oils: corn, olive, soybean, *Brassica napus* cv. Tower and *B. napus* cv. Zephyr.

Extraction and Analysis of Lipids

Rats were anesthesized with CO_2 and decapitated. The hearts were removed immediately, weighed, frozen between 2 blocks of dry ice, then pulverized and the lipids extracted as described previously (21). Total lipids were determined gravimetrically.

An Iatroscan TH-10 TLC Analyzer, Mark II (Iatron Laboratories, Inc., Tokyo, Japan; Canadian distributer: Technical Marketing Associates, Ltd., Mississauga, Ontario) was used to determine the relative composition of the cardiac lipid classes. The instrument was equipped with flame ionization detector (H_2 flow rate, 175 ml/min; air flow rate, 1850 ml/min), scanner (scanning speed, 0.47 cm/sec), integrator and recorder (sensitivity, 10 mV; chart speed, 0.42 cm/sec). A package of 10 chromarods (silica rods with a sintered coating of active adsorbent mixed with glass powder, mean thickness 75 μm) was soaked overnight in 9 N H_2SO_4. The rods were then rinsed with distilled water, dried at 100 C and prescanned twice before use. About 1 μl of total cardiac lipids dissolved in $CHCl_3/CH_3OH$ (2/1) was spotted on the rods. The rods were successively developed for a distance of 10 cm using the following solvents: (a) hexane/diethyl ether/formic acid (85:15:0.04), (b) acetone, and (c) $CHCl_3CH_3OH/H_2O$ (67:29:4). After each development, the rods were placed in an oven at 90 C for 5 min and then scanned. The rods were only partially burned from R_f 1.0 to ca. R_f 0.25 following the first and second development, and completely burned after the final development. After the first burn, mono- and diglycerides, cholesterol and the phospholipids remained; after the second burn, the phospholipids were not burned. The triglycerides had a response factor of 0.65 compared to the other lipid classes and therefore a correction factor of 1.5 was applied. The correction factors for most major components was close to unity as has been reported previously (22-24).

Total cardiac lipids (ca. 3 mg) were separated by 2-dimensional thin layer chromatography (TLC) using the solvents described by Rouser et al. (25). TLC plates were dried under N_2. Spots were visualized under ultraviolet (UV) light after spraying the plate with 2'7'-dichlorofluorescein, and scraped off the plate directly into 15-ml screw-capped tubes. Methyl esters were prepared by transesterification (21), purified on TLC and analyzed by a Hewlett Packard Model 5830 gas chromatograph, using glass columns (1.8 m x 2 mm) packed with 5% SP-2310 on 100/120 Chromosorb W AW (Supelco Inc., Bellefonte, PA). The alkenyl ethers were analyzed as described previously (26).

Analysis of variance was applied to all data. The least significant differences at the 1% level were determined from the error estimates (27).

RESULTS

Dietary oils were chosen to evaluate the effect of several fatty acids found in LEAR oils on the cardiac lipids of male rats. Soybean oil provided a similar concentration of 18:3n-3 and olive oil contained high levels of 18:1 (Table I). Corn oil was selected as an oil rich in 18:2n-6.

Male rats fed the diet containing corn oil for 16 weeks showed the best growth, whereas rats fed the other dietary oils were slightly lower (Table II). The dietary oils apparently had little effect on either the heart weight or on the total cardiac lipids, except for rats fed LEAR (cv. Zephyr) which had the lowest weight of lipid/g wet heart.

TABLE I

Fatty Acid Composition of Dietary Oils

Fatty acids	Corn	Olive	Soybean	Tower[a]	Zephyr[b]
16:0	10:9	11.6	12.4	6.1	5.3
18:0	1.7	2.5	3.7	2.0	2.3
18:1	24.3	75.5	25.4	56.5	64.7
18:2n-6	61.1	7.3	50.6	26.0	17.5
18:3n-3	0.9	0.7	7.9	7.1	5.6
20:1	0.2	0.4	-	1.5	1.5
22:1	-	0.1	-	0.3	0.9

[a]*Brassica napus* cv. Tower, a rapeseed oil low in erucic acid and glucosinolates.

[b]*Brassica napus* cv. Zephyr, a rapeseed oil low in erucic acid.

TABLE II

Body, Heart and Heart Lipid Weights, and Relative Composition of Heart Lipid

Description	Diets[a]					LSD[b]
	Corn	Olive	Soybean	Tower	Zephyr	
Body wt (g)	514	493	482	483	483	27.8
Heart wt (g)	1.34	1.25	1.27	1.35	1.39	0.12
Lipid wt (mg/g heart)	29.57	32.21	29.84	30.86	26.99	4.2
Lipid classes[c] (relative %)						
CE	1.5	1.5	2.1	1.8	1.8	0.8
TG	20.9	22.6	19.4	23.7	23.2	5.8
C	6.1	5.6	5.9	5.4	5.8	1.3
CL	8.9	8.4	8.0	7.9	7.9	1.0
PE	21.0	20.1	21.6	20.2	18.9	3.7
PS and PI	2.2	2.6	2.3	2.8	3.1	1.2
PC	34.5	33.3	34.7	32.4	32.8	4.2
SP	3.2	4.2	3.9	3.9	4.3	1.7
LPC	1.3	1.3	1.8	1.3	1.6	0.5

[a]All values are mean of 5 rats per diet, except body weight which represents the mean of 50 rats per treatment (20).

[b]LSD = least significant difference obtained from pooled error estimates of analysis of variance. Means within a row differing by more than the LSD are significantly different at the 1% level.

[c]Abbreviations: cholesterol ester (CE), triglyceride (TG), cholesterol (C), cardiolipin (CL), phosphatidylethanolamine (PE), -serine (PS), -inositol (PI) and -choline (PC), sphingomyelin (SP), and lysophosphatidylcholine (LPC).

A total analysis of the cardiac lipid subclasses was achieved with an Iatroscan using 3 separate solvent systems followed by a partial combustion technique between developments. The results are shown in Table II. There were no significant differences ($P > 0.01$) between diets in any of the lipid classes of the heart. However, diets rich in 18:1 (olive and LEAR oils) were associated with slightly higher levels of triglycerides and somewhat lower concentrations of PE and PC compared to rats fed soybean and corn oil.

The fatty acid compositions of the 2 major phospholipids of weanling rats and those fed the experimental diets for 1 and 16 weeks are given in Table III. There apparently was no effect of diet fed and age of rat on the amount (mg/g wet heart) of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Furthermore, there apparently was little effect of diet on the level of total saturated fatty acids and dimethylacetals (DMA) derived from the plasmalogenic (alkenyl ethers) compounds in these cardiac phospholipids. The concentration of monoenoic fatty acids increased significantly in rats fed diets rich in 18:1; rats fed olive oil and LEAR oil were similar. This change was rapid, i.e., after 1 week the concentration of 18:1 in cardiac PE and PC of the rat fed Zephyr oil already resembled that of the rat fed for 16 weeks.

The total level of PUFA in cardiac PE was surprisingly similar between the diets fed and time periods investigated (Table III). The major PUFA, arachidonic acid (20:4n-6), also remained fairly constant. The PUFA of PE of weanling rats were high in n-3 family acids. Dietary 18:2n-6, with little 18:3n-3 present (i.e., < 1%), rapidly substituted n-6 for n-3 PUFA, whereas a dietary level of more than 5% 18:3n-3 retained or increased the level of n-3 PUFA. Nowhere was this more evident that in the PE C22 PUFA of rats fed corn and Zephyr (Fig. 1). Although the sum of all C22 PUFA was similar between the 2 diets at both 1 and 16 weeks, the rats fed Zephyr oil were practically devoid of n-6 C22 PUFA after 16 weeks, whereas the corn-oil-fed rats had markedly reduced n-3 C22 PUFA.

The total level of PUFA in cardiac PC was slightly higher in rats fed diets rich in 18:2n-6 (corn and soybean oils) than in rats fed olive or LEAR oils (Table III). Arachidonic acid was slightly lower in rats fed LEAR oils than in rats fed corn or soybean oil, but the rats fed olive oil, with the lowest dietary level of 18:2n-6, had the highest level of 20:4n-6 and rather low levels of 18:2n-6. The C22 PUFA in cardiac PC were considerably lower than in cardiac PE. However, as in PE, the sum of the C22 PUFA was similar between the age of rats and the diets fed, and depending on the dietary level of 18:2n-6 and 18:3n-3, n-6 or n-3 C22 PUFA predominated (Fig. 1).

Linoleic acid was the major fatty acid in cardiac cardiolipin (Table IV), and the relative

TABLE III

Fatty Acid Composition of Cardiac Phosphatidylethanolamine and Phosphatidylcholine from Weanling Rats and Rats Fed Diets Containing Vegetable Oils for 1 and 16 weeks

	Phosphatidylethanolamine									Phosphatidylcholine								
		1 Week		16 Weeks							1 Week		16 Weeks					
Fatty acids[a]	0	Corn	Zephyr	Corn	Olive	Soybean	Tower	Zephyr	LSD[b]	0	Corn	Zephyr	Corn	Olive	Soybean	Tower	Zephyr	LSD
16:0 DMA[a]	5.8[d]	4.5	3.7	4.3	3.9	4.3	3.2	3.5	0.7	0.7	0.5	0.5	0.5	0.5	0.6	0.4	0.4	0.1
16:0	8.0	5.8	3.5	6.2	5.4	5.9	5.0	4.4	1.2	18.4	16.7	11.9	13.2	12.9	12.7	12.8	11.2	1.4
18:0 DMA	3.4	4.0	2.4	2.5	2.3	3.5	2.2	2.3	0.5	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.1
18:0	23.9	24.2	24.9	24.5	26.7	26.1	26.6	25.8	1.0	24.3	23.8	23.2	26.9	27.3	28.7	26.4	26.8	1.2
18:1	4.9	5.8	11.4	6.6	9.5	5.3	8.9	10.9	1.5	9.4	7.9	16.8	7.0	13.1	7.0	12.2	13.8	0.9
18:2 n-6[e]	4.3	8.0	6.2	9.4	2.3	6.7	6.0	4.9	1.8	12.0	16.5	14.8	13.3	3.9	10.5	10.5	10.0	2.8
18:3 n-3	0.2	0.1	0.6	-	-	0.3	0.4	0.3	0.1	0.1	tr	0.6	-	-	0.3	0.3	0.3	0.1
20:1	0.4	0.4	0.8	0.3	0.2	0.2	0.4	0.3	0.1	0.4	0.4	0.8	0.2	0.3	0.2	0.4	0.4	0.1
20:4 n-6	22.6	22.5	22.7	20.4	26.5	17.9	19.7	22.1	2.8	24.6	25.9	22.4	31.0	34.3	31.0	27.9	29.5	1.9
22:1	0.1	-	0.2	-	-	-	0.1	0.1	0.05	0.1	-	0.3	-	-	-	0.1	0.2	0.04
22:4 n-6	2.6	3.8	1.6	3.4	1.0	1.3	0.9	0.9	0.5	1.3	1.7	0.6	1.5	0.5	0.7	0.4	0.4	0.3
22:5 n-6	2.5	7.8	1.7	13.1	5.3	1.3	0.5	0.3	1.8	0.5	1.9	0.3	3.0	1.3	0.3	0.2	0.1	0.5
22:5 n-3	3.1	1.4	2.4	0.7	0.5	1.8	2.2	2.1	0.6	2.1	0.8	1.4	0.3	0.3	1.3	1.4	1.2	0.5
22:6 n-3	15.7	9.1	15.0	6.7	15.0	24.1	22.8	20.6	3.7	3.8	1.8	3.5	1.5	3.6	5.8	5.8	4.6	1.7
mg/g wet weight	5.3	5.6	5.1	6.2	6.5	6.4	6.2	5.1	2.3	8.9	9.5	9.2	10.2	10.7	10.4	10.0	8.9	3.4
DMA	9.2	8.5	6.1	6.8	6.2	7.8	5.4	5.8	1.1	0.9	0.7	0.7	0.8	0.8	0.8	0.6	0.6	0.2
Σ Saturates	32.7	31.2	29.4	31.4	32.4	32.3	32.1	30.6	1.4	43.7	41.5	35.8	40.5	40.4	41.5	39.5	38.2	0.8
Σ Monounsaturates	6.5	7.2	13.5	7.1	10.0	5.9	9.8	11.9	1.5	9.9	8.3	18.5	7.2	13.4	7.2	12.7	14.2	0.9
Σ Polyunsaturates	51.6	53.1	51.0	54.4	51.0	53.5	52.6	51.3	1.0	45.5	49.5	45.0	50.9	44.7	50.1	46.5	46.1	0.8
Σ n-6	32.6	42.6	33.0	47.0	35.5	27.3	27.2	28.3	3.6	39.3	46.9	39.1	49.1	40.8	43.0	39.2	40.3	2.0
Σ n-3	19.0	10.6	18.0	7.4	15.5	26.2	25.4	23.0	3.7	6.2	2.6	5.9	1.8	3.9	7.1	7.2	5.8	1.8
Σ C22 PUFA[f]	23.9	22.1	20.7	23.9	21.8	28.5	26.4	23.9	4.5	7.7	6.2	5.8	6.3	5.7	8.1	7.8	6.3	2.2

[a]Minor amounts of 14:0, 15:0, 17:0, 20:0, 20:2 and 20:3 are not included in the table.

[b]LSD = least significant difference at the 1% level (see footnote b Table II).

[c]DMA = dimethylacetal. These compounds are derived from alkenyl ethers during methylation.

[d]All values are the mean of 5 rats per diet. tr = trace (< 0.05%); (-) = no detectable amount.

[e]n-x, where n is the chain length of the fatty acids and x the number of carbon atoms from the last double bond to the terminal methyl end of the molecule.

[f]PUFA = polyunsaturated fatty acid.

concentration of this acid increased significantly compared to weanling rats on all diets except olive oil. Rats fed olive oil accumulated excessive amounts of 18:1, which were accompanied by PUFA of both the n-6 (20:4n-6 and 22:5n-6) and n-3 (22:6n-3) families to retain an average number of 2 double bonds (sum of the percentage of individual unsaturated fatty acids X number of double bonds/100) for cardiac cardiolipin. Weanling rats also contained substantial amounts of 20:4n-6 and 22:6n-3 in their cardiolipin fraction to maintain an average number of 2 double bonds. The effect of dietary 18:3n-3 was relatively minor; rats fed soybean and LEAR oil contained similar levels of n-3 PUFA.

There apparently was little difference between diets in the abundance of DMA produced during methylation (Table III). The composition of the alkenyl groups of 2 selected diets, soybean and Tower, are shown in Table V. Dietary oleic and linoleic acids influenced the alkenyl group composition; no alkenyl chains derived from linolenic or erucic acids were detected. The alkenyl group composition of the 2 phosphoglycerides was similar.

The fatty acid composition of sphingomyelin is shown in Table VI. The main difference between diets occurred with respect to the monounsaturated acids. Rats fed LEAR oils or olive oil had significantly higher levels of total monounsaturates than rats fed corn or soybean oils. The 22:1 fatty acid was found in sphingomyelin of every dietary group, but the concentration of 22:1 was significantly higher in rats fed LEAR (cv. Zephyr) than in the other groups. The position of the double bond is presumably n-9, since the long-chain monoenoic acids of sphingomyelin are formed by chain elongation from oleic (18:1n-9) acid (29).

DISCUSSION

The cardiac lipids of male rats fed LEAR oils have been investigated in the past 10 years to

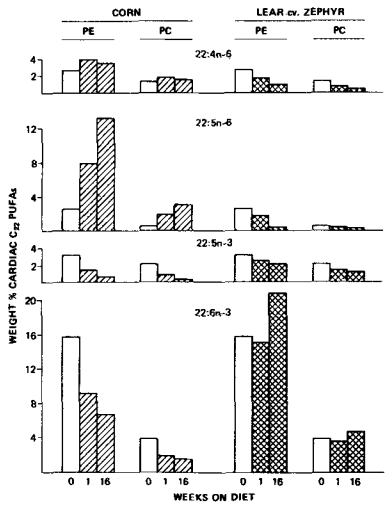

FIG. 1. The relative concentration of the C22 PUFA of cardiac phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are compared in weanling rats (open bars) with rats fed diets containing corn oil or LEAR oil cv. Zephyr for 1 and 16 weeks.

TABLE IV

Fatty Acid Composition of Cardiac Cardiolipin from Weanling Rats and Rats Fed the Experimental Diets for 1 and 16 weeks[a]

Fatty acids	Weanling	1 Week		16 Weeks					LSD
		Corn	Zephyr	Corn	Olive	Soybean	Tower	Zephyr	
16:0	4.6	2.7	2.5	2.2	2.6	1.4	1.2	1.1	0.5
18:0	3.8	3.2	2.7	1.7	2.3	1.3	0.9	0.7	0.5
18:1	10.3	8.0	12.3	4.7	22.4	4.3	7.9	9.8	2.3
18:2n-6	56.4	72.9	66.9	85.0	51.7	85.8	81.7	79.1	3.2
18:3n-3	0.5	0.1	2.3	-	-	1.3	1.6	1.7	0.2
20:1	0.8	0.3	0.7	0.1	0.5	0.1	0.4	0.3	0.1
20:4n-6	6.7	4.0	3.7	1.9	7.5	1.8	1.6	1.7	0.4
22:1	-	-	0.2	-	-	-	tr	tr	-
22:4n-6	0.7	0.8	0.5	0.3	0.2	0.1	0.1	0.1	0.1
22:5n-6	1.1	0.9	0.4	0.9	2.5	0.1	tr	tr	0.1
22:5n-3	1.3	0.7	0.8	0.1	0.2	0.3	0.3	0.3	0.2
22:6n-3	5.7	1.8	2.5	0.4	5.2	1.5	1.5	1.8	0.7
Av. no of double bonds[b]	2.15	1.99	1.99	1.91	2.02	1.98	1.94	1.94	

[a]See footnotes to Table III.

[b]Average number of double bonds is the sum of the percentage of individual unsaturated fatty acids X number of double bonds/100.

TABLE V

Composition of the Alkenyl Groups from Rat Heart Ethanolamine and Choline Phosphoglycerides

Chain length: no. of double bonds	Ethanolamine phosphoglycerides		Choline phosphoglycerides	
	Soybean	Tower	Soybean	Tower
14:0	2.0	1.6	3.0	2.9
15:0	2.2	1.1	2.6	3.3
16:0	24.2	24.4	20.8	14.4
16:1	2.2	2.1	2.5	3.1
17:0	2.7	1.4	3.2	3.9
17:1	1.2	1.1	1.3	2.0
18:0	36.6	35.9	38.8	36.3
18:1	13.5	23.0	18.0	26.2
18:2	14.8	8.8	9.8	7.8

TABLE VI

Fatty Acid Composition of Sphingomyelin from Hearts of Rats Fed Vegetable Oils for 16 Weeks

Fatty acids	Diets[a]					LSD[b]
	Corn	Olive	Soybean	Tower	Zephyr	
14:0	0.5	0.6	0.8	0.9	1.0	0.5
15:0	0.4	0.5	0.5	0.7	0.5	0.3
16:0	13.4	13.1	12.5	11.4	14.5	2.8
17:0	0.6	1.1	0.7	0.8	0.6	0.6
18:0	11.5	12.4	12.6	12.5	14.3	2.2
18:1	1.6	2.9	1.4	2.1	2.6	1.6
19:0	1.7	1.2	1.0	1.9	1.5	0.7
20:0	24.9	19.7	15.9	21.0	20.0	2.3
21:0	1.3	1.6	2.0	0.9	0.9	0.3
22:0	16.1	15.7	26.4	21.6	17.6	2.8
22:1	0.2	0.5	0.4	0.5	0.9	0.3
23:0	4.5	4.6	5.2	2.5	2.4	0.6
ECL 23.6[c]	2.9	2.8	2.9	1.3	1.5	0.9
24:0	13.9	10.7	12.1	8.6	8.4	1.6
24:1	5.9	11.2	5.8	12.3	12.5	2.0
X:1[d]	7.7	14.6	7.6	14.9	16.0	

[a]Values are means of 5 rats per diet.

[b]LSD = least significant differences at the 1% level.

[c]ECL = equivalent chain length (28).

[d]X:1, monounsaturated fatty acids.

determine whether myocardial damage in rats fed dietary LEAR oils is related to changes in lipid classes or their fatty acid composition. Cardiac neutral lipids, particularly the triglycerides, were altered readily both qualitatively and quantitatively with different dietary fatty acids (30). However, these triglyceride changes have been discounted as the cause of the necrotic heart lesions as first assumed (31). The reason was that female rats of the same strain (Sprague-Dawley) (32) and male rats of another strain (Chester Beatty) (33) had similar cardiac triglyceride changes without causing the subsequent long-term necrotic heart lesions (32,33). Furthermore, the cardiac triglyceride accumulation was attributed to the dietary C22 long-chain monoenoic fatty acids (9,32).

The cardiac phospholipids, on the other hand, being mainly membrane constituents (13), have been found to be relatively resistant to quantitative changes; no changes had been observed in the total level of cardiac phosphorus from dietary oils (34). The results of this study demonstrated an in vivo regulation in the concentration of all cardiac phospholipids irrespective of the age of the rat (weanling 3-week-old, 1 and 16 weeks on diet) and source

of dietary oils. Only a slightly lower level of PE was noted in rats fed one of the 2 LEAR oils (cv. Zephyr). Blomstrand and Svensson (17) also observed decreased values of cardiac PE in rats fed diets containing low levels of erucic acid. No significant differences were observed in the level of sphingomyelin between rats fed LEAR oils or any other vegetable oil, similar to that reported by Dewailly et al. (18), and contrary to that reported by Beare-Rogers (35,36). The concentration of lyso-PC was low (1.8-2.5% recalculated as relative % of total phospholipids from Table II), and only traces of free fatty acids, and mono- and diglycerides were detected, indicating little, if any, autolysis of cardiac lipids during the extraction procedure employing an improved extraction technique (21). This was in marked contrast to lyso-PC values of 5-10% (% of total phospholipids) reported by Beare-Rogers et al. (14) using conventional extraction techniques. Extraction of cardiac lipids from rats fed high-fat diets are particularly susceptible to autolysis during conventional homogenizations, giving rise to high values of free fatty acids and lyso-PC (21).

As noted in the results of this study, there also was an apparent in vivo regulation in the fatty acid composition of all major cardiac phospholipids. In PE and PC, the total level of saturates and polyunsaturates remained fairly constant irrespective of the age of the rat and the dietary oil fed; the monounsaturates showed evidence of a linear relationship to dietary 18:1 ($r \geqslant 0.87$). Among the polyunsaturates, there was little change in 20:4n-6 and the total C22 PUFA. However, among the C22 PUFA, there were wide differences between n-3 and n-6 fatty acids, depending on the dietary concentration of 18:3n-3 or 18:2n-6, or including members of these fatty acids families as may be the case in weanling rats (37).

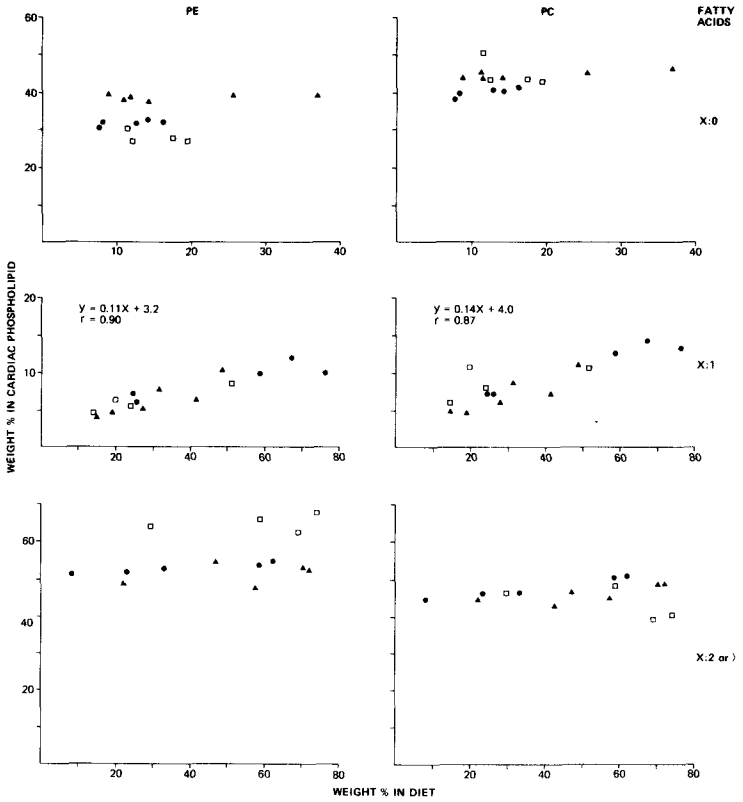

FIG. 2. The results of this study (•) were combined with those of 2 other publications (14 [▲] and 38 [□]). The relative concentration of dietary saturated (X:0), monounsaturated (X:1) and polyunsaturated (X:2 or >) fatty acids (abscissa) were plotted against the corresponding levels of these fatty acids in cardiac phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (ordinate). The relationship of monounsaturated fatty acids is represented by the linear equation, $y = ax + b$, where, a is the slope of the line, b the intercept, and x and y the concentration of monounsaturates in the diet and phospholipids, respectively. The correlation coefficient is r.

The results of this study were combined with those of 2 other studies (14,38) in which male rats were fed high-fat diets and cardiac PE and PC were analyzed to provide an even wider range of dietary fatty acids. The results are plotted in Figure 2. Dietary saturates (X:0), ranging from 8 to 37%, apparently had no effect on the concentration of saturates in cardiac PE or PC. On the other hand, a 5-fold difference in the dietary level of monounsaturates (X:1), from 14 to 76%, resulted in only a doubling of this group of fatty acids in either phospholipid. The biochemical mechanism of excluding monounsaturates from cardiac PE and PC may explain why 20:1 and 22:1 were not incorporated extensively into the 2 cardiac phospholipids, even when diets rich in these fatty acids were fed to rats (33). Finally, the total level of polyunsaturates (X:2 or >) remained fairly constant despite widely different levels of dietary 18:2n-6 (7-73%) and 18:3n-3 (trace to 53%). Among the PUFA, there was a small response of cardiac levels of 18:2n-6 to dietary 18:2n-6 (PE, $y = 0.13x + 6.3$, $r = 0.29$; PC, $y = 0.09x + 5.4$, $r = 0.64$). The concentration of 20:4n-6 remained fairly constant in PE and PC. Dietary levels of 5-14% 18:3n-3 depressed 20:4n-6 only slightly in PC, and it required a high dietary level of 18:3n-3 (52.6% in linseed oil) to depress 20:4n-6 significantly in both PE and PC.

The greatest change, however, was seen among the C22 PUFA (Fig. 3). In rats fed as little as 5% dietary 18:3n-3, most of the C22 PUFA consisted of the n-3 family, whereas the C22 PUFA of the n-6 family predominated in diets poor in 18:3n-3. The change in the C22 PUFA with diet were already evident after 1 week on the selected diets, corn and LEAR (cv. Zephyr) oils. The C22 PUFA of the n-3 family also predominated in weanling rats because the n-3 family acids are bioconcentrated in the fetus during its development (37). In addition, it suggests that the diet used by the supplier may have been rich in n-3 family acids. The results of Gudbjarnason et al. (39,40) and Rocquelin (15) show a similar replacement of n-3 for n-6 family acids in the total cardiac phospholipids by feeding diets containing 18:3n-3 (linseed oil, ref. 15) or n-3 family acids (cod liver oil, ref. 39).

Therefore, according to the results, dietary 18:3n-3 up to 15% apparently did not inhibit the conversion of 18:2n-6 to 20:4n-6, but the subsequent conversion to 22:4n-6 and 22:5n-6 was much reduced in favor of desaturation and elongation of 18:3n-3. Such a protective mechanism of maintaining a certain proportion of 20:4n-6 has also been observed in erythrocytes of premature infants (41).

Regulation of the fatty acid composition was also evident in cardiac cardiolipins, a component present in mitochondrial membranes (13). The substitution of monounsaturates in cardiolipin of rats fed olive oil apparently has been compensated for by the incorporation of PUFA to maintain a similar average number of double bonds of ca. 2. Monounsaturates have an apparent affinity for cardiac cardiolipin as demonstrated previously by Blomstrand and Svensson regarding 22:1n-9 (17).

It is quite evident from the results of this study that the incorporation of 18:1 and the substitution of n-3 for n-6 C22 PUFA into cardiac phospholipids was related to the dietary concentration of these fatty acids, and was not peculiar to any specific oil. At the present time, it is impossible to estimate the effect of these changes in cardiac phospholipids on the membrane structure and function, although it is known that specific phospholipids, and often certain fatty acids in these phospholipids, are required for full activity of several membrane-bound enzymes (13,37).

Evidence from this laboratory supports the concept that rats fed high-fat diets, particularly those rich in 18:3n-3, developed cardiac membranes which were "more fragile" than those

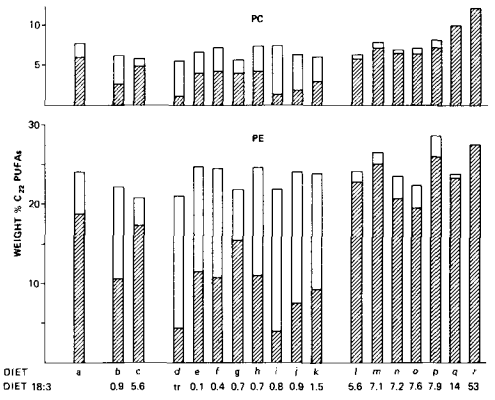

FIG. 3. The relative concentration of the n-3 (solid bar) and n-6 (open bar) C22 PUFA of weanling rats (a) and rats fed different dietary fats and oils for 1 (b and c) or more than 8 weeks (d to r). The dietary level of 18:3n-3 is given. The diets are: corn (b and j); Zephyr (c and 1); peanut (d) (38); lard/corn, 3:1 mixture (e) (14); poppy seed/lard/corn, 4:3:1 mixture (f) (14); olive (g); poppy seed (h) (14); safflower (i) (38); sunflower (k) (14); Tower (m); poppy seed/Tower, 1:1 mixture (n) (14); soybean (o) (38); soybean (p); Tower (q) (14); and linseed (r) (38) oils. Abbreviations: PC = phosphatidylcholine; PE = phosphatidylethanolamine; PUFA = polyunsaturated fatty acids.

from rats fed laboratory chow. Dow-Walsh et al. (42) showed that rats fed diets rich in monoenes and 18:3n-3 had heart mitochondria which aged much faster. It was also found that substantially higher levels of free fatty acids and lyso-PC (21) were produced during the isolation of cardiac lipids particularly from rats fed diets rich in 18:3n-3 using conventional extraction techniques. This suggests that the membranes are more easily dissociated and subject to autolysis before enzymes are inhibited by chloroform/methanol. A "more fragile" membrane could be subject to greater cellular breakdown which could lead to focal necrosis. It is therefore notable that oils containing appreciable amounts of 18:1 (e.g., olive [2,7, 8,20] or peanut oils [43-46]), 18:3n-3 (e.g., soybean [3,4,6,20,46]) or linseed oils [7,8]) or 18:1 and 18:3n-3 (e.g., LEAR oils (2-4,6-10, 14,16,20,33,44-46]) apparently are associated with heart lesions in male rats if fed these oils at a high level in the diet for prolonged periods of time. In fact, both 18:1 and 18:3n-3 were positively correlated with heart lesions in male rats in a statistical analysis carried out on a large number of published results involving cardiopathological examinations in male rats (5).

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: VI. Methyl 9,15- and 12,15-octadecadienoate

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ABSTRACT

The gas chromatography-mass spectrometry (GC-MS) method developed in preceding papers was extended to the structural analysis of autoxidation products of methyl *cis*-9,*cis*-15-octadecadienoate and of an 87% concentrate of *cis*-12,*cis*-15-octadecadienoate. Eight isomeric hydroxydienes with one allylic and one isolated double bond were identified from oxidized 9,15-diene and 2 conjugated hydroxydienes from oxidized 12,15-diene, after reduction of the hydroperoxides. The proportions of 16- (15%) and 17-hydroperoxides (22%) from 9,15-diene were significantly higher than that of the other isomers (8-12% each: 8-, 9-, 10-, 11-, 14- and 15-OOH). Similarly, the amount of 16-hydroperoxide from 12,15-diene was larger (42%) than the 12-hydroperoxide (31%). Substantial amounts of dihydroxy esters with one OH substituent on carbons-8, -9, -10 or -11 and the other OH on carbons-14, -16 or -17, were identified after hydrogenation in highly oxidized 9,15-diene. The implications of these hydroperoxide analyses are discussed in relationship to the precursors of flavor deterioration of oils and partially hydrogenated oils containing an ω-3 double bond.

INTRODUCTION

Isomeric dienes known as isolinoleate with double bonds separated by more than one methylene group are formed by partial hydrogenation of linolenate in soybean oil (1-4). These dienes have one of their double bonds located between C-14 and C-16 and have been implicated as precursors of flavor deterioration (2,5-8). The availability of *cis,cis*-9,15- and 12,15-octadecadienoates (9) made it possible to elucidate the mechanism of this deterioration. In previous papers of this series, isomeric hydroperoxides were characterized and determined quantitatively by gas chromatography-mass spectrometry (GC-MS) of the hydroxystearate derivatives from autoxidized oleate, linoleate, linolenate and their mixtures (10-12). In this paper, the same GC-MS method was applied to the study of oxidation products of pure *cis*-9,*cis*-15- and of an 87% concentrate of *cis*-12-,*cis*-15-octadecadienoate.

EXPERIMENTAL PROCEDURES

The *cis,cis*-9,15- and 12,15-dienes were prepared from hydrazine-reduced methyl linolenate according to the Butterfield et al. procedure (9). The 9,15-diene fraction obtained by argentation-countercurrent distribution ($AgNO_3$-CCD) was 96% pure by GC. To obtain 100% pure 9,15-diene, the 9,12- and 12,15-diene impurities were removed by selective oxidation as follows. After treating a sample with O_2 at 60 C to a peroxide value (PV) of 160, it was chromatographed through a short silicic acid column. Pure *cis,cis*-9,15-diene was eluted with *n*-hexane, leaving the oxidized diene impurities on the column. The 12,15-diene concentrate was prepared from the CCD fraction containing 9,12-diene by recycling 5 times with the 200-tube $AgNO_3$-CCD system (9). The portion of the diene fraction containing more than 50% 12,15-diene isomer was used at each cycle. GC analysis of the final concentrate showed: 87.3% 12,15-, 11.4% 9,12- and 1.3% 9,15-diene. The all-*cis* configuration of these dienes was established by capillary GC (13).

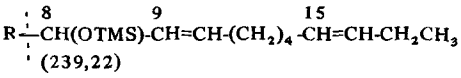
The same procedures as reported previously were used for autoxidation, analyses, catalytic hydrogenation, $NaBH_4$ reduction, silylation and GC-MS (10). Samples having PV below 300 were concentrated as previously described (14). For quantitative standardization of the GC-MS method, the same authentic 10-, 12-, 13- and 16-hydroxyoctadecanoates were used as before (10-12). Authentic 14-, 15- and 17-hydroxyoctadecanoates were prepared by Raney nickel hydrogenation of the corresponding keto esters synthesized by literature methods (15). In these syntheses, ω-bromocarboxylic acids were used instead of the corresponding iodo acids in the alkylation of the appropriate β-keto esters. A sample of methyl ester of the naturally occurring 17-L-hydroxyoctadecanoic acid (16) was generously donated by Dr. A. P. Tulloch (Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada).

RESULTS

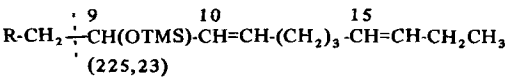
Eight isomeric allylic hydroxydienes are formed by reducing the corresponding hydro-

peroxides of 9,15-diene. The structure of the trimethylsilyl (TMS) derivatives and the mass fragment characteristic of the noted cleavage (m/e, relative abundance in percent) are as follows:

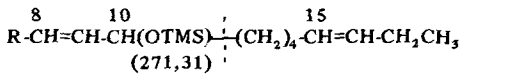
[A]



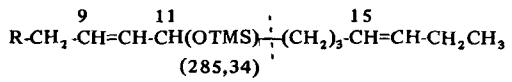
[B]



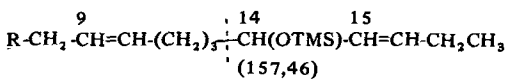
[C]



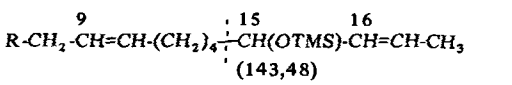
[D]



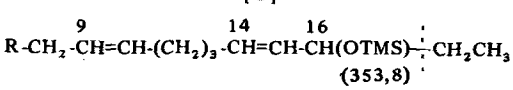
[E]



[F]



[G]



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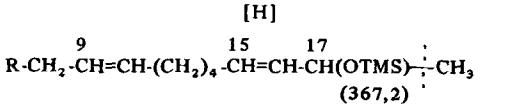
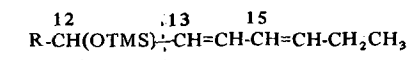


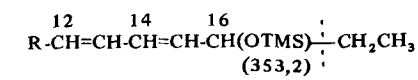
The relative intensity of the mass fragments just indicated cannot be interpreted quantitatively because of complications resulting from allylic rearrangements (12). Also, m/e 157 from isomer E and m/e 143 from isomer F are more intense because the mass fragments of the corresponding allylic isomers G and H are not favored and tend not to form the high-energy methyl and ethyl radicals.

The TMS derivatives of the 12- and 16-hydroxydienes formed by reducing the corresponding hydroperoxides of 12,15-diene are shown next with the mass fragmentation data.

[I]



[J]



Mass fragment m/e 353 for hydroxydiene J is minor and unfavored for the same reason suggested for G, because it tends not to form the high-energy ethyl radical. The 9,12-diene impurity (11.4%) produced only minor mass fragments corresponding to 9- and 13-hydroxydienes (m/e 225 and 311 with relative abundances 3 and 1, respectively).

Confirmative and additional structural information were obtained by GC-MS analyses of oxidized samples after catalytic hydrogenation and silylation. The GC-MS computer-generated

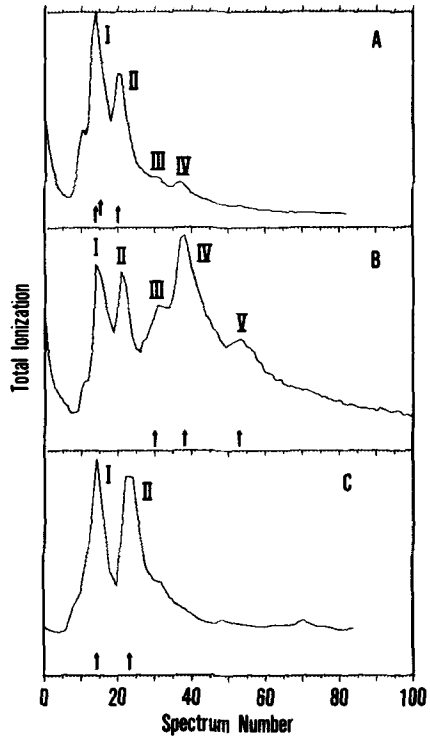


FIG. 1. Gas chromatography-mass spectrometry total ion traces of trimethyl silyl ethers from hydroxy derivatives of hydrogenated-autoxidized dienes. A: 9,15-Diene; peroxide value, 608. B: 9,15-Diene; peroxide value, 1247. C: 12,15-Diene; peroxide value, 264. Vertical arrows indicate individual spectra given in Table I.

total ions chromatograms showed that in the oxidized 9,15-dienes, peak I results from a mixture of 8-, 9-, 10-, 11- and 14-OTMS stearates, and peak II to a mixture of 15-, 16- and 17-OTMS esters (Fig. 1A, Table I). Partial separation of isomers is indicated by comparing mass fragments of 14-OTMS in spectra no. 14 and 15. Peaks III, IV and V became more prominent at higher levels of oxidation (PV 1247) (Fig. 1B). MS showed the presence of di-OTMS esters with one hydroxy substituent at C-8, C-9, C-10 or C-11 and the other at C-14, C-16 or C-17 (Table I). In the oxidized 12,15-diene, peak I results mainly from the 12-OTMS ester and peak II from the 16-OTMS ester (Fig. 1C, Table I).

Quantitative GC-MS analyses were previously standardized with synthetic samples of 8-, 9-, 10- and 11-hydroxystearates from oxidized oleate (10), 9- and 13-hydroxystearates from oxidized linoleate (11) and 9-, 12-, 13- and 16-hydroxystearates (TMS derivatives) from linolenate (12). For this study, the quantitative GC-MS method was further checked with artificial mixtures of authentic 10-, 12-, 13-, 14-, 15-, 16- and 17-hydroxystearates (TMS derivatives). A bias was observed for the values of the 14-hydroxystearate. A consistently higher percentage of this isomer than theoretical values arises from an artifact at m/e 159 (one of the diagnostic ions for 14-OTMS stearate) evident with the corresponding TMS derivatives of pure synthetic methyl 15-, 16- and 17-hydroxystearate isomers. To correct for this bias, a computer program was used based on a least square technique (17) to solve 8 linear equations developed from standard spectra run with the pure 14-, 15-, 16- and 17-hydroxystearates (TMS derivatives). The calculated mean standard deviation between known and experimental compositions in 6 mixtures was 2.2 (Table II).

Samples of 9,15- and 12,15-dienes were oxidized to different peroxide values and at different temperatures. The isomeric hydroxy ester compositions remained remarkably constant at different levels of oxidation (Table III). The oxidized 9,15-dienes gave, on one hand, about equal distribution of hydroxy isomers on the 8-, 9-, 10- and 11-carbon positions (10-12%). On the other hand, there was a significantly higher proportion of the 16- and

TABLE I

Mass Spectral Data on Hydrogenated-oxidized Dienes after Silylation

Peak (spectrum no.)	Characteristic fragments m/e (relative abundance)	Identification[a] (C-18 OTMS methyl esters)
	9,15-diene (PV 608)	
Figure 1A		
I (14)	243 (68.0), 245 (95.3)	8-OH
	229 (71.5), 259 (70.4)	9-OH
	215 (72.2), 273 (57.4)	10-OH
	201 (100), 287 (64.5)	11-OH
	159 (31.4), 329 (7.9)	14-OH
I (15)	159 (100), 329 (32.3)	14-OH
II (20)	145 (100), 343 (6.9)	15-OH
	131 (100), 357 (25.8)	16-OH
	117 (100), 371 (8.2)	17-OH
	9,15-diene (PV 1247)	
Figure 1b		
III (30)	245 (12.3), 259 (19.4), 273 (8.7), 287 (14.5)	8, 9, 10, 11-x[b]
	159 (28.3), 131 (22.2), 117 (24.6)	x-14, 16, 17
IV (38)	245 (17.6), 259 (15.8), 273 (9.9), 287 (10.7)	8, 9, 10, 11-x
	159 (10.2), 131 (36.5), 117 (37.6)	x-14, 16, 17
V (53)	245 (10.2), 259 (20.3), 273 (7.2), 287 (6.7)	8, 9, 10, 11-x
	159 (13.1), 131 (38.5), 117 (19.2)	x-14, 16, 17
	12,15-diene (PV 264)	
Figure 1C		
I (14)	187 (100), 301 (71.9)	12-OH
II (23)	131 (100), 357 (58.9)	16-OH

[a]Based on comparison with reference compounds and reported fragmentation schemes (10-12, 17).

[b]8-x means OH on C-8 on ester side of molecule, x-14 means OH on C-14 on hydrocarbon side of molecule.

TABLE II

Quantitative GC-MS Analysis of Synthetic Hydroxystearates (TMS Derivatives)

Known mixtures	Relative percent[a]							Standard deviation
	10-OH	12-OH	13-OH	14-OH	15-OH	16-OH	17-OH	
Mixture 1		40.0				60.0		
Found		39.5				60.5		0.5
Mixture 2		50.0				50.0		
Found		54.6				45.4		4.6
Mixture 3	15.0			20.0	10.0	25.0	30.0	
Found	13.1			22.3	10.9	24.9	28.8	1.5
Mixture 4				15.0	15.0	35.0	35.0	
Found				14.8	16.0	30.7	38.4	2.8
Mixture 5				25.0	25.0	25.0	25.0	
Found				27.4	26.4	21.8	24.4	2.1
Mixture 6	14.2	14.2	14.2	14.2	14.2	14.2	14.2	
Found	17.1	13.2	11.9	15.6	15.0	12.4	13.9	1.7
Found	17.4	13.4	11.8	15.0	14.6	12.2	14.9	1.7
Mean standard deviation								2.2

[a]Based on computer summation of masses 215 + 273 for 10-OH isomer, masses 187 + 301 for 12-OH isomer, masses 173 + 315 for 13-OH isomer, masses 159 + 329 for 14-OH isomer, masses 145 + 343 for 15-OH isomer, masses 131 + 357 for 16-OH isomer and masses 117 + 371 for 17-OH isomer (TMS derivatives). Analyses of 14-OH, 15-OH, 16-OH, and 17-OH were corrected for the contribution of m/e 159 in the spectra of these isomers by a computer program (17).

TABLE III

Quantitative GC-MS Analysis of Isomeric Hydroxystearates from Autoxidized 9,15- and 12,15-Dienes (TMS Derivatives)

Peroxide value	Temperature, C	Relative percent[a]									
		8-OH	9-OH	10-OH	11-OH	12-OH	13-OH	14-OH	15-OH	16-OH	17-OH
						9,15-Diene					
75	25	12	12	10	10			11	8	17	20
120	25	10	13	12	10			11	9	16	19
262	25	11	12	10	10			11	8	16	22
287	60	12	12	10	10			12	8	14	22
558	60	12	11	10	7			12	7	17	24
608	60	12	11	10	10			11	7	15	23
1112	60	12	12	10	10			11	8	15	22
1247	60	11	12	11	9			12	6	15	23
302	80	12	12	12	10			10	9	13	22
601	80	9	13	11	10			12	9	14	22
973	80	11	12	10	10			11	7	15	24
Average		11	12	11	10			11	8	15	22
						12,15-Diene					
420	25	0.1	8	2	2	31	9	6	1	38	3
770	25	0.1	5	0.3	1	34	8	6	2	42	1
264	60	0.1	6	0.5	2	31	7	5	1	44	3
645	60	0.3	7	1	2	31	7	5	2	40	4
1104	60	0.1	6	1	3	30	7	5	1	44	3
Average			6	1	2	31	8	6	1	42	3

[a]See footnote a in Table II.

17-hydroxy isomers relative to the 14- and 15-hydroxy isomers. Although the Δ15 double bond of 9,15-diene is oxidized ca. 12% more than the Δ9 double bond (56 vs 44%), there is a marked preferential attack on carbons 16 and 17 (37%).

Oxidation of the 12,15-diene concentrate produced the hydroxystearate isomers corresponding to the 12- and 16-hydroperoxides expected from 12,15-diene and those corresponding to the 9- and 13-hydroperoxides from the 11.4% 9,12-diene present in the concentrate (Table III). The average composition shows that the hydroxy esters derived from the 12,15-diene are composed of 31% 12- and 42% 16-isomers. The other hydroxy esters include 14% 9- and 13-isomers arising from 9,12-diene, and the rest from 9,15-diene. These results show that in the 12,15-diene, a preferential oxidation occurs at C-16 relative to C-12.

DISCUSSION

This study of hydroperoxides from dienoic fatty esters containing an ω-3 double bond can provide a better understanding of the precursors of volatile compounds producing off-flavors in linolenate-containing oils. The terminal pentene radical in linolenate, CH_3-CH_2-CH=CH-$CH_2^{\cdot}$- has been associated with the unique flavor deterioration known as "flavor reversion" in soybean oil (18). Oxidation of the ω double bond in isolinoleate, which is found in partially hydrogenated soybean and linseed oils, also produces the so-called "hardening" flavor (5-7). The aldehyde associated with this unpleasant flavor was identified as 6-nonenal and shown to be derived from either 9,15- or 8,15-dienes (7).

The mechanism for the free radical oxidation of methyl oleate was previously reviewed (10). Assuming the same mechanism for the Δ15 double bond of 9,15-diene, the formation of allylic radicals is suggested between C-15 and C-17 on one hand, and between C-14 and C-16 on the other hand. Oxygen attack on either end of these 3-carbon radicals produces a mixture of allylic 14-, 15-, 16- and 17-hydroperoxides (Fig. 2). Oxidation of the Δ9 double bond occurs as in oleate to produce a mixture of 8-, 9-, 10- and 11-hydroperoxides. This study identified the 8 isomeric hydroperoxides from 9,15-diene as the TMS derivatives of the hydroxy dienes A-H. Further, selective oxygen attack was shown on C-16 and C-17 to give significantly higher concentrations of the corresponding 16- and 17-hydroperoxides (Table III). Therefore, the end positions of the allylic radicals are not equivalent and the external C-16 and C-17 are more reactive with oxygen than the internal C-14 and C-15. At high degrees of oxidation, (PV 1247) 9,15-diene produces a significant amount of dihydroxy esters (Fig. 1B). Assuming that these dihydroxy esters are derived from dihydroperoxides, we deal with a mixture in which one hydroperoxide is located on carbons-8, -9, -10 or -11, and the other hydroperoxide on carbons-14, -16 or -17 (Table I).

The free radical oxidation of the 12,15-diene proceeds as in methyl linoleate (11) and produces a mixture of conjugated 12- and 16-diene hydroperoxides through a pentadienyl radical between C-12 and C-16. These diene hydroperoxides were identified as the TMS hydroxy dienes I and J. As in the 9,15-diene, we observe a selective oxygen attack on the C-16 producing significantly more 16- than 12-diene hydroperoxides (Table III).

With linolenate, oxidation of the ω double bond also appears favored since the 16-hydroperoxide is formed in significantly higher proportion (46%) than the other isomers (31% 9-, 12% 13- and 11% 12-hydroperoxides) (12). The analogy with 12,15-diene is complicated because with linolenate, the internal 12- and 13-hydroperoxides may tend to cyclize into endoperoxide hydroperoxides (12). However, the significantly higher proportion of the 16-hydroperoxide than the 9-hydroperoxide is consistent with the conclusion made with the 12,15-diene that the carbon of the pentadienyl radical closest to the end of the fatty acid chain is most reactive with oxygen.

Selective oxygen attack on the ω-carbons of 9,15- and 12,15-dienes has important implications in the formation of flavor deterioration of linolenate-containing oils with similar unsaturation (8). The 10-hydroperoxide diene arising from the 9,15-diene (corresponding to

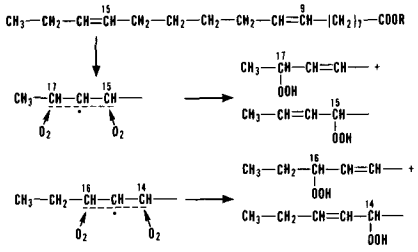


FIG. 2. Mechanism of autoxidation of Δ15 double bond of 9,15-diene.

hydroxydiene C) was suggested as the precursor of 6-nonenal identified in oxidized 9,15- and 8,15-dienes (7). Synthetic 6-*cis* and 6-*trans*-nonenal were reported to have a flavor reminiscent of green melon (6). In our current work, we also detected a melony or cucumber odor in highly oxidized samples of 9,15-diene. Keppler et al. (6,7) found other carbonyl compounds that were unidentified in oxidized hydrogenated linseed and soybean oils, but they reported that the 6-*trans*-nonenal had the most intense odor and taste. The corresponding aldehydes expected from the 8-, 9- and 11-hydroperoxides (corresponding to A, B and D) are, respectively: 2,8-undecadienal, 2,7-decadienal and 5-octenal (8). From the 14-, 15-, 16- and 17-hydroperoxides (corresponding to E, F, G and H), the expected aldehydes are, respectively: 2-pentenal, 2-butenal, propional and acetaldehyde. From the 12- and 16-hydroperoxides of 12,15-diene (corresponding to I and J), the expected aldehydes are 2,4-heptadienal and propional. In addition to these carbonyl compounds, various hydrocarbons and alcohols would be expected (8). Decomposition of secondary oxidation products such as the dihydroperoxides, identified indirectly in highly oxidized 9,15-diene, will further complicate the composition of volatile compounds contributing to flavor and odor of oils. A more complete identification of the volatile products formed in the oxidation of these model dienes will yield a more complete understanding of the flavor deterioration in oils containing an ω-3 double bond.

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Intercorrelations among Plasma High Density Lipoprotein, Obesity and Triglycerides in a Normal Population[1]

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ABSTRACT

The interrelationships among fatness measures, plasma triglycerides and high density lipoproteins (HDL) were examined in 131 normal adult subjects: 38 men aged 27-46, 40 men aged 47-66, 29 women aged 27-46 and 24 women aged 47-66. None of the women were taking estrogens or oral contraceptive medication. The HDL concentration was subdivided into HDL_{2b}, HDL_{2a} and HDL_3 by a computerized fitting of the total schlieren pattern to reference schlieren patterns. Anthropometric measures employed included skinfolds at 3 sites, 2 weight/height indices and 2 girth measurements. A high correlation was found among the various fatness measures. These measures were negatively correlated with total HDL, reflecting the negative correlation between fatness measures and HDL_2 (as the sum of HDL_{2a} and $_{2b}$). Fatness measures showed no relationship to HDL_3. There was also an inverse correlation between triglyceride concentration and HDL_2. No particular fatness measure was better than any other for demonstrating the inverse correlation with HDL but multiple correlations using all of the measures of obesity improved the correlations. Partial correlations controlling for fatness did not reduce any of the significant correlations between triglycerides and HDL_2 to insignificance. The weak correlation between fatness and triglycerides was reduced to insignificance when controlled for HDL_2.

INTRODUCTION

Growing evidence supports a possible protective role for serum high density lipoproteins (HDL) against atherosclerotic cardiovascular disease (ASCVD) (1-7). The evidence is based largely on epidemiologic studies in which only total HDL or HDL cholesterol was measured. Although heterogeneity of HDL has long been recognized, the subdivisions of HDL into HDL_2 and HDL_3 have recently been reexamined and redefined by Anderson et al. (8). They concluded that the "protective" effect of HDL resides in the more rapidly floating fraction, HDL_2 and its subfractions HDL_{2b} and HDL_{2a}. Krauss et al., using statistical methods to subdivide HDL, arrived at the same conclusion (9). Anderson further demonstrated that most of the variability of total HDL and HDL cholesterol is accounted for by HDL_2 (10).

Two factors known to be inversely related to HDL are obesity (3,6,11,12) and serum levels of very low density lipoproteins (VLDL) or triglycerides (TG) (3,5,10-14). Obesity and TG, in turn, are known to be correlated (11,15,16). Anderson reported that the negative correlation between VLDL and HDL was entirely explained by the negative correlation between VLDL and HDL_2 (10).

The role of obesity in the relationship between plasma triglycerides and HDL is not known, nor has the relationship between obesity and the newly redefined HDL components yet been determined. Data from a study of plasma lipids and lipoproteins in a group of residents of Modesto, CA (17), made possible an examination of the intercorrelations among obesity, triglycerides, HDL and HDL subfractions measured by analytical ultracentrifugation in an adult population.

METHODS

Subjects

The study population was a random subsample of a larger group of residents of Modesto, CA. The details of the population and the cholesterol, triglyceride and lipoprotein concentrations by age and sex have been previously reported (17). The obesity measurements are reported here for the first time. The sample for this study was a group of 160 persons, 40 men and 40 women aged 27-46, and 40 men and 40 women aged 47-66 (17). Of this group, 29 were excluded from this analysis since measurements on 2 men and 1 woman were not complete, and 26 women had been taking estrogen or birth control pills. This left a total population of 131.

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Plasma Lipids and Lipoproteins

Plasma lipid and lipoprotein measurements obtained and reported previously (17) were used for the calculations reported here. The blood for lipid and lipoprotein analysis was drawn at a variable time during the morning after overnight fasting and a light fat-free breakfast (black coffee or tea, dry toast and orange juice). The selection of subjects was randomized with respect to day of examination and time of blood sampling (17). Plasma triglycerides and total cholesterol were determined by Technicon Autoanalyzer (AAII) techniques already explained (18).

The procedure for measurement of lipoproteins, including VLDL and HDL, by analytic ultracentrifugation and the methods used for analysis of the schlieren pattern and computerized corrections for baseline, concentration, Ogston-Johnson and viscosity effects have been described (19).

HDL components were analyzed by the Anderson et al. method (8). The corrected HDL schlieren curve of material floating at a density of 1.20 can be quantitatively represented as an array of heights, representing concentrations, along the $F^0_{1.20}$ 0-9 flotation rate scale. This array can then be treated statistically in various ways. Classically, the summation of the intervals $F^0_{1.20}$ 0-3.5 has been denoted as HDL_3 whereas $F^0_{1.20}$ 3.5-9 has been denoted HDL_2. Anderson et al. (8) have redefined the subdivision of HDL. Briefly, according to this method the array of heights is statistically resolved into 3 overlapping curves denoted as HDL_{2b}, HDL_{2a} and HDL_3 in order of increasing density, by computerized fitting of reference schlieren patterns to each individual curve. The reference schlieren patterns in turn had been derived from the analytic ultracentrifugation of 3 HDL components isolated by equilibrium density gradient ultracentrifugation of plasma from normal men and women. The 3 major components of HDL separated by this method fell within the density intervals 1.063-1.100, 1.100-1.125 and 1.125-1.200. These density limits thus approximate the density characteristics of HDL_{2b}, HDL_{2a} and HDL_3.

Anderson's subdivision of HDL resulted in a shift of some material previously included in HDL_3 into HDL_{2a}. According to his data, the flotation rate $F^0_{1.20}$ 3.5 usually taken as the upper limit of HDL_3, is too high and includes some HDL_2. Unless stated otherwise, HDL_3 in this paper indicates HDL_3 defined by Anderson et al., and HDL_2 indicates the sum of HDL_{2b} and HDL_{2a} as defined by Anderson et al. (8).

In order to provide additional verification for the new subdivision, the Krauss et al. method was also used to subdivide the HDL schlieren pattern (9). Their subdivision was based on the results of correlation coefficients between HDL and LDL subgroups. They found that the correlations between LDL subgroups and HDL of $F^0_{1.20}$ 0-1.5 were opposite in sign from the correlations with HDL of $F^0_{1.20}$ 2-9, the crossover occurring between $HDL^0_{1.20}$ 1.5 and 2. Accordingly, in that study and in this study, HDL was divided into 2 subgroups by the summation of $F^0_{1.20}$ flotation intervals 0-1.5, denoted $HDL^{0-1.5}$, and $F^0_{1.20}$ 2-9, denoted HDL^{2-9} (9). Correlations were carried out between these HDL subgroups (9) and the HDL subgroups measured by the Anderson et al. techniques (8).

Anthropometric Measurements

Height, weight, girth at the waist and girth at the iliac crest were recorded. Skinfold thickness was measured at 3 sites (scapular [scap], triceps and forearm) using Lange skinfold calipers. Body index ($wt/ht^2 \times 100$) and ponderal index ($ht/\sqrt[3]{wt}$) were calculated from weight in pounds and height in inches.

Statistics

Simple correlation coefficients were calculated between triglycerides, each measurement of fatness and total HDL and each of its components. Multiple correlations and forward regressions were also carried out using each lipid measurement in turn as the dependent variable, and all of the obesity measurements as independent variables (20). Partial correlations also were carried out, controlling for obesity or for single lipid variables.

RESULTS

Table I gives the mean anthropometric measures, plasma triglycerides and cholesterol for the 4 age-sex groups. Women had greater triceps and forearm skinfold thickness than did men of the same age group ($p < 0.001$).

Simple Pearson correlation coefficients showed that the obesity measures were interrelated (Table II). The 2 body indices, ponderal index and body index, were highly (and inversely) intercorrelated in all 4 age-sex groups (r=0.93-0.97) as were the 2 girths (r=0.81-0.91). The correlation was negative because ponderal index decreases whereas body index increases with increasing fatness. Girths were rather highly correlated with indices (/r/= 0.56-0.91). Of the skinfolds, scap was generally most highly correlated with the indices (/r/= 0.62-0.85) and girths (/r/=0.59-0.82). Scap also

TABLE I

Anthropometric Characteristics and Plasma Lipids (means and SD)[a]

Group	Skinfolds (cm)			Circumferences (cm)		Indices[b]		Triglycerides (mg/dl)	Cholesterol (mg/dl)
	Scapular	Triceps	Forearm	Waist	Iliac	Body index	PI[c]		
Men 27-46 N=38	18.2 ± 7.2	15.5 ± 4.9	5.1 ± 1.6	89.9 ± 8.4	99.5 ± 7.7	3.60 ± 0.39	12.59 ± 0.45	115.8 ± 53.2	194.9 ± 30.3
Men 47-66 N=40	20.0 ± 7.3	15.5 ± 6.5	4.9 ± 1.4	92.1 ± 8.4	101.7 ± 8.0	3.67 ± 0.48	12.43 ± 0.52	141.8 ± 72.3	229.1 ± 33.3
Women 27-46 N=29	18.3 ± 7.9	28.7 ± 9.1	7.8 ± 3.6	74.0 ± 8.8	94.6 ± 9.4	3.32 ± 0.54	12.60 ± 0.65	73.5 ± 20.9	186.7 ± 29.9
Women 47-66 N=24	21.5 ± 9.7	29.1 ± 9.9	8.2 ± 3.0	78.7 ± 9.4	96.7 ± 7.0	3.42 ± 0.42	12.37 ± 0.52	134.9 ± 99.1	233.5 ± 39.3

[a]Blood for lipid analysis was obtained after a light fat-free breakfast.

[b]Body index = weight/height^2.

[c]PI = ponderal index, height/$\sqrt[3]{\text{weight}}$.

TABLE II

Pearson Correlation Coefficients between Various Measures of Body Fatness

	Iliac	Forearm	Triceps	Scap	PI[a]	Body[b] index	Iliac	Forearm	Triceps	Scap	PI	Body index
	Males 27-46 N=38						Males 47-66 N=40					
Waist	0.91	0.73	0.69	0.76	-0.78	0.91	0.88	0.50	0.57	0.59	-0.74	0.80
Iliac		0.70	0.64	0.65	-0.61	0.80		0.63	0.62	0.72	-0.81	0.89
Forearm			0.58	0.56	-0.61	0.74			0.69	0.58	-0.45[d]	0.45[d]
Triceps				0.63	-0.51	0.60				0.74	-0.55	0.58
Scap					-0.63	0.68					-0.62	0.65
PI						-0.93						-0.97
	Females 27-46 N=29						Females 47-66 N=24					
Waist	0.90	0.14[e]	0.59	0.81	-0.81	0.90	0.81	0.41[c]	0.60[d]	0.78	-0.56[d]	0.64
Iliac		0.26[e]	0.66	0.78	-0.78	0.88		0.49[c]	0.80	0.82	-0.73	0.64
Forearm			0.68	0.26[e]	-0.41[c]	0.35[e]			0.58[d]	0.38[e]	-0.58[d]	0.54[d]
Triceps				0.64	-0.74	0.73				0.85	-0.76	0.77
Scap					-0.83	0.85					-0.76	0.80
PI						-0.96						-0.95

[a]PI = ponderal index, $ht/\sqrt[3]{wt}$, pounds, inches.

[b]Body index = $wt/ht^2 \times 100$.

All $p < 0.001$ except as noted by superscripts: [c]< 0.05; [d]< 0.01; [e]Not significant.

was correlated with triceps (r=0.63-0.85) and less strongly with forearm (r=0.25-0.58). In all 4 age-sex groups, scap was the skinfold most highly correlated with body index, followed in order by triceps and forearm. Waist circumference, body index and scap were selected as representative of the 3 types of fatness measures: girth, height-weight indices and skinfolds, for calculating the relationships between fatness and lipid fractions.

The correlation between HDL^{2-9} and HDL_2 was greater than 0.99 ($p < 0.001$) in all 4 age-sex groups. Moreover, the correlation between HDL_3 and $HDL^{\delta-1.5}$ was between 0.96 and 0.97 ($p < 0.001$) for the 4 age-sex groups.

The correlations between TG and VLDL were 0.94, 0.92, 0.72 and 0.98 (all $p < 0.001$) for the younger and older men and younger and older women, respectively (not shown). Correlations with TG thus reflect correlations with VLDL. The lower correlation for younger women is possibly explained by imprecision of VLDL measurement at low levels of VLDL. Triglycerides will be used in the analyses to follow.

Table III shows the simple (Pearson) correlation coefficients between selected anthropometric measures and HDL components, total HDL and triglycerides. One or more fatness measures were inversely correlated with HDL_{2b}, HDL_{2a}, and with total HDL in most age-sex groups and, for the group as a whole; the correlations with HDL_{2b}, HDL_{2a} and total HDL were similar and were strongest for younger men and older women. Fatness measures were not correlated significantly with HDL_3 in any group except for a positive correlation (r=0.27, $p < 0.01$) with waist circumference in the group as a whole. The correlations between height and HDL subfractions were inconsistent; height was inversely correlated with total HDL in young men but directly correlated in young women.

The negative correlation seen between fatness measures and total HDL could be entirely accounted for by the negative correlation between each measure and HDL_{2b} and HDL_{2a}, the correlation with HDL_{2b} being slightly stronger. Total HDL was almost as

TABLE III

Pearson Correlation Coefficients between Selected Obesity Measures, Height, HDL and Its Fractions and Triglyceride

	Scap	Waist	Body index	Triglyceride	Height
HDL_{2a}	-0.39[a]	-0.28	-0.25	-0.42[b]	-.31
	-0.20	-0.24	-0.12	-0.25	-.04
	-0.03	0.04	-0.02	-0.40[a]	.37[a]
	-0.58[b]	-0.53[b]	-0.26	-0.73[c]	.07
	-0.23[b]	-0.49[c]	-0.26[b]	-0.42[c]	-.44[c]
HDL_{2b}	-0.38[a]	-0.36[a]	-0.30	-0.46[b]	-.26
	-0.32[a]	-0.33[a]	-0.31[a]	-0.20	-.13
	-0.32	-0.26	-0.33	-0.44[a]	.49[b]
	-0.43[a]	-0.28	-0.22	-0.48[a]	.13
	-0.27[b]	-0.47[c]	-0.37[c]	-0.33[c]	-.32[c]
HDL_3	-0.06	-0.04	-0.03	0.34[a]	-.19
	0.00	0.10	0.15	0.03	.04
	0.14	0.10	0.06	0.28	-.23
	0.14	0.07	0.00	0.33	-.45[a]
	0.08	0.27[b]	0.16	0.33[c]	.07
Total HDL	-0.40[a]	-0.32[a]	-0.28	-0.31	-.36[a]
	-0.27	-0.26	-0.16	-0.22	-.07
	-0.25	-0.19	-0.16	-0.46[a]	.51[b]
	-0.55[b]	-0.44[a]	-0.29	-0.59[b]	-.04
	-0.27[b]	-0.46[c]	-0.31[b]	-0.32[b]	-.41[c]
Triglyceride	0.23	0.22	0.30		
	0.01	0.11	0.20		
	-0.08	-0.07	-0.06		
	0.57[b]	0.74[c]	0.27		
	0.26[b]	+.38[c]	0.25[b]		

Order: Men 27-46 (N-38); men 47-66 (N-40); women 27-46 (N-29); women 47-66 (N-24). All men and women (N-131).

[a] $p < 0.05$.

[b] $p < 0.01$.

[c] $p < 0.001$.

satisfactory as HDL_{2a} or HDL_{2b} for showing the negative relationship between HDL and scap.

Triglyceride concentration was significantly inversely correlated with HDL_{2b}, HDL_{2a}, or both except in older men (Table III). Cholesterol (not shown) was not related to HDL_2 but in the group as a whole both cholesterol and triglycerides were directly correlated with HDL_3.

The correlations between triglycerides and fatness were weak and significant only for older women and for the group as a whole.

Since HDL_2 and triglycerides were the lipid fractions most clearly related to fatness, further statistical analysis is reported only for these fractions. Multiple correlations and forward regressions were carried out using all 7 fatness measures as the independent variables and either triglycerides or HDL_2 (the sum of HDL_{2b} and HDL_{2a}) as the dependent variable. Table IV shows the maximal significant multiple r and r^2 and the sign of the partial slope of the individual fatness measurements which contributed to the maximal significant r^2 at the 5% level. Since no significant correlations for triglycerides were observed in older men or younger women, data on these relationships are omitted from the table. However, their inclusion in the "all" category did not reduce the contribution of any of the fatness variables to insignificance. The strongest statistical effect of fatness measurements was in younger men and older women. For the group as a whole, the multiple correlations indicated that fatness accounted for 25% of the variance ($r^2 \times 100$) of triglycerides ($p < 0.001$) and 32% of the variance of HDL_2 ($p < 0.001$).

The magnitude of the individual contribution of each fatness variable to total r^2 of triglycerides and HDL_2 was difficult to assess since the fat measurements were highly intercorrelated, and the order varied for each age-sex group. However, the direction of the effect of the fatness variables showed certain patterns of interest. The slope of the regression of triglycerides on scap was always positive and that of HDL_2 and scap always negative. Although in simple correlations the limb skinfolds correlated little if at all with triglycerides or HDL_2, (not shown), triceps and/or forearm skinfold bore a relationship to triglycerides and HDL_2 opposite to that of scap, i.e., negative for triglycerides, positive for HDL_2, evident in most groups shown in Table IV. The trend was strongest for older women in whom scap contributed 28 (-) and limb skinfolds together 5% (+) of the variance of HDL_2 (not shown). Although the 2 circumferences, when significant, were positively related to triglycerides, they varied in sign in their relationship to HDL_2 and not always in the same direction. The sign of each index was always the same as the sign of the other. Since body index is directly related whereas PI is inversely related to fatness (or bulk), the effects of fatness as indicated by the 2 indices were opposite to each other for both triglycerides and HDL_2 in all groups in which both indices appeared.

Partial correlations were carried out control-

TABLE IV

Multiple Correlations and Regressions of HDL_2 (HDL_{2b} and $_{2a}$) or Triglycerides on All Seven Fatness Measures[a]

	Triglycerides			HDL_2				
	Men	Women	All men;	Men		Women		All men;
	(27-46)	(47-66)	all women	(27-46)	(47-66)	(27-46)	(47-66)	all women
Scap	+	+	+	-			-	-
Triceps	-	-	-	(+)		(-)	+	+
Forearm	(-)	+	-	+		+	(+)	+
Waist		+	+	+	(-)	-	+	-
Iliac		+	+	-			-	-
PI	-	-	-	-		+	+	
Body index		-	-	-		+	+	+
Multiple r	0.45	0.90	0.50	0.59	0.31	0.58	0.71	0.56
Multiple r^2	0.20	0.80	0.25	0.35	(0.09)	0.33	0.56	0.32
p	0.05	0.001	0.001	0.05	(0.054)	0.05	0.05	0.001

[a]The maximum significant r^2 ($p < 0.05$) and the sign of the slope of fatness variables that contributed to it are shown. Absence of a sign indicates that the fatness measure did not contribute significantly to r^2. Where the measure contributed to r^2 with $p > 0.05$ but < 0.10, the sign is in parentheses, but the measure was not included in the r^2 listed. Since there were no significant correlations between triglyceride and any fatness measure (alone or in combination) for older men and younger women, data on these are omitted from the table but are included in the "all" category.

ling for triglycerides, for HDL_2 (as HDL_{2b+2a}) and for scap. The results are shown in Table V. Controlling for triglycerides slightly reduced the significant correlations between HDL_2 and scap but the correlation was reduced to insignificance only in older women. Controlling for scap slightly decreased the correlations between triglycerides and HDL_2 but none of the significant correlations was reduced to insignificance. Controlling for HDL_2 eliminated the few significant correlations between scap and triglycerides. Controlling for height (not shown) had no effect on any significant correlation.

DISCUSSION

The well known inverse relationship between plasma HDL concentration and fatness was shown in this study to be entirely accounted for by HDL_2 and its subfractions, HDL_{2b} and HDL_{2a} as defined by Anderson et al. (8). Fatness had no correlation or at best only a weak positive correlation with HDL_3. There was little difference between HDL_{2b} and HDL_{2a} in showing the negative correlation with obesity. For epidemiologic studies, then, total HDL_2 or for that matter total HDL thus adequately reflects the correlation between obesity and HDL.

As previously reported for VLDL (10), plasma triglyceride concentration was negatively correlated with HDL_2 and positively correlated with HDL_3. This positive correlation is at variance with the older literature, reviewed by Anderson et al. (8), in which HDL_3 tended to share with HDL_2 a negative correlation with VLDL. Anderson et al. (8) using physicochemical techniques, redefined the subdivision of HDL into its component parts, HDL_2 and HDL_3, and presented evidence for 2 HDL_2 subfractions, HDL_{2b} and HDL_{2a} (see Methods). The reclassification resulted in a shift of the most rapidly floating portion of HDL_3 into HDL_{2a} as newly defined (8). For the first time a positive correlation between HDL_3 and VLDL was observed (10), suggesting that the negative correlation previously observed may have resulted from contamination of HDL_3 with HDL_2. The nature of the correlation between HDL_3 and VLDL in various hyperlipidemic states and in other populations remains to be determined.

Krauss et al. (9) found that the correlation between VLDL and HDL material falling between the flotation intervals $F^0_{1.20}$ 0-1.5 ($HDL^{0-1.5}$) (see Methods) was positive whereas the correlation with HDL material in the flotation intervals $F^0_{1.20}$ 2-9 (HDL^{2-9}) was negative. The very high intercorrelations between HDL_{2b+2a} and HDL^{2-9} and between HDL_3 and $HDL^{0-1.5}$ shown in this study indicate that, for statistical purposes, HDL^{2-9} reflects HDL_{2b+2a} and $HDL^{0-1.5}$ reflects HDL_3. Thus, the Anderson et al. physiocochemical data (8) and the Krauss et al. statistical data (9) suggest that the flotation range of HDL_3 is closer to $F^0_{1.20}$ 0-1.5 than to the older definition $F^0_{1.20}$ 0-3.5 and is thus more narrow than that classically defined.

Although fatness and triglycerides were both significantly inversely correlated with HDL_2 in the group as a whole, they were only slightly correlated with each other, the correlation being strongest for younger men and older women, and for the group as a whole. While the

TABLE V

Simple Correlations and Partial Correlations between Scap, HDL_2 (as HDL_{2a} and $_{2b}$) and Triglycerides[a]

	Men		Women		All men: all women
	(27-46)	(47-66)	(27-46)	(47-66)	
HDL_2 with Scap					
Simple	-0.41[b]	-0.28	-0.27	-0.53[c]	-0.28[c]
Controlled for TG	-0.37[b]	-0.29	-0.35	-0.30	-0.19[b]
HDL_2 with TG					
Simple	-0.46[c]	-0.24	-0.50[c]	-0.62[d]	-0.40[d]
Controlled for Scap	-0.42[c]	-0.25	-0.54[c]	-0.45[b]	-0.35[d]
Scap with TG					
Simple	0.23	0.01	-0.08	0.57[c]	0.26[c]
Controlled for HDL_2	0.03	-0.06	-0.25	0.36	0.17

[a]2-tailed significance test was used for the partial correlations.

[b]$p < 0.05$.

[c]$p < 0.01$.

[d]$p < 0.001$.

postprandial sampling might account for the weakness of the correlation, similar low-grade correlations between triglycerides and fatness have been widely reported in the literature (11,15-16). Inability to measure fat mass accurately may also be responsible for the weakness of the correlations with triglycerides. There is no generally satisfactory method for measuring total body fat. The cumbersome densitometric method based on underwater weighing is generally thought to be the most accurate and correlates well with single or multiple skinfold measurements (21). Bray et al. found that scapular skinfold thickness, despite its substantial error of measurement, was second best of 8 measurements in reflecting weight loss of obese women undergoing weight reduction with drug therapy (22). It is possible that skinfold measurements are preferable to densitometric methods for estimating body fat, since the constancy of composition and density of the lean body mass on which the densitometric method depends has been challenged (23,24). In this study, the 3 skinfolds were the fat measures most frequently retained in the multiple correlations as contributing to the effect of fatness on triglycerides and HDL_2.

The improvement of the correlations between fatness measures and both triglycerides and HDL_2 in this study could have resulted from the better estimate of fatness afforded by multiple measurements. Another possibility is that some specific features of fatness were uncovered by the multiple correlations. The contribution of the limb skinfolds was usually opposite in sign to that of scap, suggesting that the preponderance of central over peripheral obesity characteristic of adult-onset obesity (15,25) favors high triglycerides and low HDL_2. A similar conclusion was drawn for triglycerides in an earlier study (15). These data even suggest a slight protective effect of fatness of extremeties against low HDL_2. The physiological significance of central obesity may also be different from that of peripheral obesity, as shown in the Framingham study by a positive correlation between scapular skinfold thickness and risk of nonmyocardial infarction ischemic heart diseases, but inverse correlation of such risk with arm skinfold thickness (26).

In the multiple correlations, the effects on triglycerides and HDL_2 of body bulk assessed by body index was opposite from that assessed by PI. Height or some other aspect of lean body mass, reflected to a different degree by the 2 indices, could be important in the regulation of triglycerides and HDL_2.

The reason for the stronger correlations between some fatness measures and HDL fractions in younger men and older women than in older men and younger women is unknown. Sampling bias with uneven distribution of environmental and other factors known to influence HDL and triglycerides is possible. A high correlation between fatness and lipid fractions in older women similar to that observed here was noted in the Framingham study (11). Possibly younger men and older women are undergoing weight gain. Bierman reported a later rise and decline in weight with advancing age in women compared to men (27).

Partial correlations were carried out to test the possibility that since fatness and triglycerides were both negatively correlated with HDL_2, fatness might contribute to the observed correlation between triglycerides and HDL_2. Controlling for fatness (scap) only slightly decreased the significance of correlations between triglycerides and HDL_2 and none was rendered insignificant. The correlation between fatness and HDL_2 was only slightly reduced by controlling for triglycerides except in older women in whom the significant correlation was abolished. For the remaining groups, fatness was more strongly associated with HDL_2 than with triglycerides. Controlling for HDL_2 abolished the few weak significant intercorrelations between triglycerides and scap. It thus appears likely that the relationship between fatness and triglycerides was dependent on the relationship of each with HDL_2.

The mechanism for the statistical effect of fatness and of triglycerides on HDL_2 is uncertain. A role for HDL as a receptor of surface components released by degradation of VLDL and chylomicrons has been postulated (28). The increase in HDL free cholesterol and phospholipids with increasing total HDL levels (29) and with increasing HDL_2 (30) is consistent with the hypothesis that brisk catabolism of VLDL, by providing essential components for HDL_2, is associated with low triglycerides and high HDL_2, as is the positive correlation between HDL and adipose tissue lipoprotein lipase (31). Impaired triglyceride catabolism would account for the association between elevated triglycerides and low HDL_2. The role of HDL_3 in this system is unknown. Possibly it is a precursor of HDL_2 (32).

The basis for the relationship of HDL_2 with fatness is unknown, but the insulin resistance of large adipose cells of adult-acquired obesity (33) and consequent decreased activity of lipoprotein lipase, an insulin-sensitive enzyme, could be a factor. Since the relationship was statistically independent of the inverse correlation between triglycerides and HDL_2, decreased HDL_2 could be a more sensitive sign of

subtle impairment of VLDL or chylomicron removal than is plasma triglyceride concentration.

Considering the multitude of factors possibly affecting HDL levels, e.g., age, sex, sex hormones, activity, smoking and alcohol (34), the effect of obesity measured by multiple correlations was remarkably large. For the group as a whole, nearly one-third of the variance of HDL_2 was accounted for by multiple fatness measures. It may be that better definition of obesity will further increase the variability of HDL accounted for by degree of obesity.

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Effect of Different Fatty Acids on Triacylglycerol Secretion in Isolated Rat Hepatocytes

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ABSTRACT

Lipoprotein triacylglycerol secretion was studied in isolated rat hepatocytes incubated with different albumin-bound fatty acids and labeled glycerol. The release of labeled triacylglycerol was stimulated more by unsaturated fatty acids than by saturated ones. When lipoprotein secretion was related to cell triacylglycerol synthesis, an effect of unsaturation was no longer observed. Instead the secretion rate, expressed in this manner, increased with increasing fatty acid chain length. For the first time, the secretion of molecular species of triacylglycerol has been studied. The distribution of labeled glycerol among different species was the same in the cells and in the secreted product, indicating that different triacylglycerols were secreted without selectivity. It is concluded that the fatty acid structure influences lipoprotein triacylglycerol secretion and it is emphasized that the effects observed depend on the method of quantitation of the secretion rate.

INTRODUCTION

Fatty acids are secreted from the liver mainly as triacylglycerol in very low density lipoproteins (VLDL). The rate of VLDL secretion varies considerably in different nutritional and metabolic states and increased VLDL production is encountered in some types of hyperlipemia. Studies with perfused liver have shown that increased availability of fatty acids to the liver dramatically stimulates VLDL secretion (1,2). Previously, we showed that hepatocytes isolated after collagenase perfusion are capable of lipoprotein secretion (3). In this system, fatty acids stimulated lipoprotein secretion but so far the effect of different fatty acids had not been investigated.

Dietary intake of different fatty acids markedly affects lipoprotein composition and turnover (4), although the mechanisms behind these changes remain unclear. One possibility is that the stimulatory effect of fatty acid on lipoprotein secretion from the liver is dependent on fatty acid structure. Previous studies indicated that all long-chain fatty acids were well incorporated into lipoproteins secreted by the liver (2), but preliminary studies in our laboratory suggested that completely saturated triacylglycerols were secreted at a lower rate (5). Therefore, the secretion of different molecular species of triacylglycerol from isolated hepatocytes has been studied in detail. The results suggest that both fatty acid unsaturation and chain length influence lipoprotein triacylglycerol secretion, depending on the method for measurement of secretion rate.

EXPERIMENTAL

Materials

Radiochemicals were obtained from the Radiochemical Centre, Amersham, U.K. Collagenase, type I, was obtained from Sigma, St. Louis, MO. Bovine serum albumin was obtained from Serva, Heidelberg, FRG, and was defatted according to Chen (6).

Preparation and Incubation of Hepatocytes

Hepatocytes were prepared from male Sprague-Dawley rats fed a balanced diet ad libitum as described previously (7,8). The cells were incubated in duplicate in 1 ml of Hanks' buffer containing 10 mM phosphate (pH 7.4), 2% albumin, an amino acid mixture described by East et al. (9), albumin-bound fatty acids as indicated and usually 10 nmol [1(3)-^{3}H]-glycerol. The mixtures were incubated at 37 C in 25-ml Erlenmeyer flasks by shaking at 60-80 strokes/min. Blank incubations lacking cells were included in the experiments. After incubation, the cells and the medium were separated by low-speed centrifugation as described previously (3). In preliminary experiments, this procedure sometimes gave too high a measure of lipoprotein secretion, since the medium also contained some membrane fragments. Therefore, the low-speed supernatant was routinely centrifuged at 100,000 x g for 40 min in a MSE 50 ultracentrifuge.

Lipid Analyses

Lipids were extracted from the cells and the medium, and the extracts were washed as described previously (3). They were separated by thin layer chromatography (TLC) on Silica Gel H with diethyl ether/petroleum ether/acetic acid (30:70:1) as developing solvent. Radioactivity in triacylglycerol, fatty acid, diacylglycerol and polar lipids was measured by liquid scintillation (10). Triacylglycerols isolated by TLC were also separated into molecular species

by argentation TLC (11). Protein was determined according to Lowry et al. (12) using human serum albumin as standard.

RESULTS

Effect of Fatty Acid Concentration

In hepatocytes incubated with 10 nmol [^{3}H]glycerol, the maximal amount of ^{3}H in lipid was attained after ca. 30 min and thereafter underwent little change for 1 hr (3). After an initial lag, secretion of [^{3}H]triacylglycerol started; 90 min was a suitable incubation time. Less than 5% of lipid ^{3}H was found in the fatty acid moieties. Oleic acid stimulated triacylglycerol secretion from isolated hepatocytes (Fig. 1). In most experiments, 0.25-0.75 mM oleic acid gave near maximal stimulation, whereas 1 mM oleic acid was inhibitory. In a few experiments, 1 mM oleic acid also stimulated lipoprotein secretion, whereas triacylglycerol synthesis in the cell was always markedly stimulated by increasing concentrations of fatty acid, as observed previously (10). The low secretion in the presence of 1 mM oleic acid (Fig. 1) was therefore not secondary to inhibition of triacylglycerol synthesis. Instead, at high concentrations, the free fatty acid itself probably interfered with some step in lipoprotein assembly or secretion. The reason for the variable response to 1 mM fatty acid in our experiments is unknown. Perhaps isolated hepatocytes are more susceptible to such a toxic effect than the intact liver and the perfused liver. It recently was observed also in cultured hepatocytes (13). Perfusion of liver with high concentrations of fatty acids does not inhibit lipoprotein secretion (1,2).

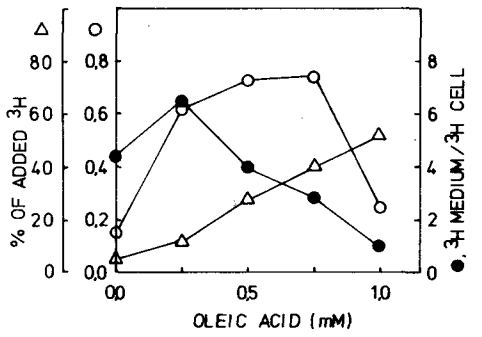

FIG. 1. Effect of oleic acid on lipoprotein secretion in isolated hepatocytes. Isolated hepatocytes were incubated for 90 min with [^{3}H]glycerol and albumin-bound oleate as described in Experimental. Symbols: % of added ^{3}H recovered in cell lipids (△) and triacylglycerol of the medium 100,000 X g supernatant (○); ●, 100 X (lipid ^{3}H in medium supernatant)/^{3}H in cell lipid.

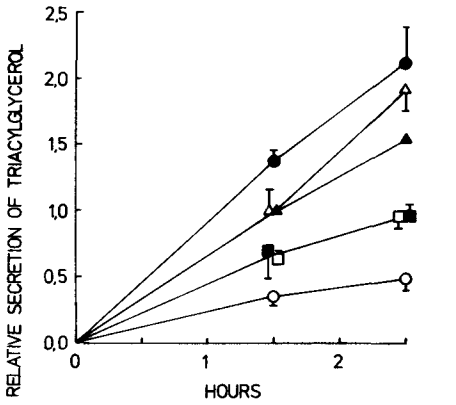

FIG. 2. Effect of different fatty acids on lipoprotein secretion in isolated hepatocytes. Isolated hepatocytes were incubated with 0.25 mM fatty acid as described in Fig. 1 and the amount of ^{3}H in triacylglycerol of the medium 100,000 X g supernatant was determined. The value obtained in each experiment after 90 min incubation with 0.25 mM oleate was set at 1.0 and other data were calculated relative to this. Data are means (SE) from 3 experiments. Symbols: ○, no fatty acid; □, laurate; △, myristate; ■, stearate; ●, palmitoleate; ▲, oleate.

Effect of Different Fatty Acids

Lipoprotein secretion from hepatocytes incubated with 0.25 mM or 0.5 mM fatty acids was then investigated. The relative effects of different fatty acids were similar at both concentrations and therefore only the results with the lower concentration are presented (Figs. 2 and 3). The mean secretion of [^{3}H]-triacylglycerol at 90 min, expressed as the percentage of added ^{3}H was: laurate–0.43; myristate–0.64; palmitoleate–0.98; stearate–0.45; oleate–0.70; and control without fatty acid–0.19. This absolute amount of secreted [^{3}H]triacylglycerol varied somewhat between different experiments but the relative effect of different fatty acids was always the same. To facilitate the comparison between fatty acids, the amount of [^{3}H]triacylglycerol in the 100,000 x g supernatant after incubation with 0.25 mM oleic acid for 90 min was set at 1.0 and other data were then normalized relative to this value (Fig. 2).

All fatty acids tested stimulated triacylglycerol secretion. Palmitoleate, myristate and oleate were the most stimulatory, whereas laurate and stearate were less effective (Fig. 2). This indicates that, when measured in this way, lipoprotein secretion was stimulated more by unsaturated fatty acids than by saturated ones. The secretion measured as the release of [^{3}H]-triacylglycerol may, however, depend to some extent on the total incorporation of [^{3}H]-

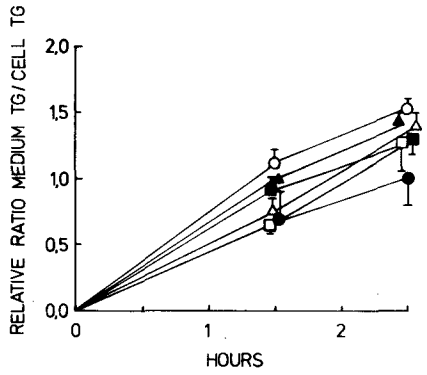

FIG. 3. Lipoprotein secretion relative to cell triacylglycerol. radioactivity. The experiments were performed as described in Fig. 2 and the ratio medium triacylglycerol ^3H/cell triacylglycerol ^3H was calculated. The value in each experiment obtained from incubations with 0.25 mM oleate at 90 min was set at 1.0 and other data were calculated relative to this. Symbols are as in Fig. 2.

glycerol into cell tricylglycerol. Several experiments with [^{14}C]-labeled fatty acids showed that the uptake into cell lipids, mainly triacylglycerol, increased with chain length for saturated fatty acids (Table I). Similar differences were seen in the incorporation of [^3H] glycerol into cell triacylglycerol. At 90 min, the incorporation was: (0.25 mM) laurate–4.79; myristate–6.85; palmitoleate–15.3; stearate–3.21; oleate–5.49; and control without fatty acid–1.37% of added ^3H.

To obtain a measure of triacylglycerol secretion independent of differences in cell triacylglycerol synthesis, the ratio [^3H]-triacylglycerol in medium supernatant/cell [^3H] triacylglycerol was calculated (Fig. 3). To facilitate a comparison between fatty acids, the ratio obtained in each experiment after incubation with 0.25 mM oleic acid for 90 min (mean ratio 0.146) was set at 1.0 and other data were calculated relative to this. When presented in this way, the data indicate smaller differences between different fatty acids than those shown in Figure 2. The relative triacylglycerol secretion increased with acyl chain length but no influence of unsaturation could be observed. Thus, the selective effects of different fatty acids on triacylglycerol lipoprotein secretion measured as release of newly synthetized triacylglycerol can be explained by at least 2 mechanisms in this system. First, differences in cell uptake and triacylglycerol synthesis from different fatty acids give rise to variable amounts of cell triacylglycerol, the precursor of lipoprotein triacylglycerol. Second, when these factors have been taken into account, the fatty acyl chain length apparently influences the secretion by some other mechanism.

TABLE I

Lipid Synthesis from Different Fatty Acids in Isolated Rat Hepatocytes[a]

[^{14}C] Fatty acid	0.25 mM	0.5 mM
Linoleate	31.0	21.6
Myristate	37.1	40.3
Palmitate	52.1	53.4
Oleate	34.2	33.7
Linoleate	48.9	50.7

[a]Isolated hepatocytes were incubated for 90 min with the indicated [^{14}C] fatty acid (0.25 or 0.5 mM) as described in Experimental. The incorporation into cellular lipids is expressed as the percentage of added ^{14}C (means from 2 experiments).

Secretion of Molecular Species of Triacylglycerol

Administration of a single fatty acid to liver preparations will change the fatty acid composition of hepatic triacylglycerol. This holds for the perfused liver (1,2) and also for suspended hepatocytes (data not shown). The added fatty acid would also be expected to dominate in secreted lipoprotein triacylglycerol, as observed in perfused liver (14). It is unknown, however, whether any molecular species of triacylglycerol is secreted preferably. Therefore, the distribution of radioactivity among triacylglycerols of different degrees of unsaturation was compared in cells and medium (Table II). The data for cells and medium were very similar indicating that labeled cell triacylglycerol species were secreted without selectively. The distribution of molecular species among cell triacylglycerols (Fig. 4) and medium triacylglycerols was relatively constant at different incubation times, and therefore the comparison in Table II is not complicated by any time-dependent changes.

As communicated previously, some of the experiments indicated that saturated triacylglycerols were secreted to a lesser degree than unsaturated ones, when hepatocytes were incubated with 1 mM saturated fatty acid (5). This may indicate that there is an upper limit for the amount of saturated triacylglycerol, which can be accomodated within the lipoprotein particles secreted from the liver. The influence of such a concept in vivo when a mixture of fatty acids is taken up by the hepatocyte cannot be assessed until further experimental evidence is available.

TABLE II

Effect of Different Fatty Acids on the Secretion of Different Molecular Species of Triacylglycerol from Isolated Rat Hepatocytes[a]

Triacyl-glycerol source	No. of double bonds in triacylglycerol		Fatty acid addition: Laurate	Myristate	Palmitate	Stearate	Oleate
Medium	0	3.3	36.7	28.5	22.5	6.4	0.0
	1		21.0	29.4	28.6	22.6	15.9
	2	6.2	10.9	10.5	19.8	30.9	
	3a	6.0	5.6	9.2	5.3	15.2	43.3
	3b	14.1					7.0
	4	5.0	26.0	22.4	23.7	24.9	33.8
	> 4	65.4					
Cells	0	2.1	39.0	31.5	21.6	7.1	1.9
	1		20.3	26.9	29.2	22.9	18.7
	2	7.7	9.1	13.4	19.2	30.6	
	3a	5.3	8.6	5.8	7.5	14.6	42.3
	3b	14.6					5.6
	4	4.9	23.1	22.5	22.4	24.8	31.6
	> 4	65.3					

[a]Hepatocytes were incubated for 90 min in Hanks' buffer containing amino acids (as specified in Experimental), 2% albumin, 0.25 mM fatty acid and [^{3}H]glycerol. Triacylglycerols from cells and medium were separated according to unsaturation by argentation chromatography and the percentage distribution of ^{3}H among molecular species of triacylglycerol is shown in the Table. Data represent 3-5 experiments.

DISCUSSION

Free fatty acid taken up from plasma is the major precursor of VLDL-triacylglycerol secreted by the liver (2), although fatty acids synthetized within the liver also may contribute (15). Also, in humans on a high-carbohydrate diet, a significant proportion of VLDL-fatty acid was synthetized within the liver (16). In individuals consuming fat-rich Western-type diets, the major VLDL precursor would therefore be plasma fatty acid. Dietary fatty acids rapidly appear as plasma fatty acid (17), and furthermore, a dietary change from saturated to unsaturated fat decreases serum triacylglycerol as well as serum cholesterol (4). It was therefore hypothetized that such a change in plasma fatty acid composition influences hepatic VLDL-secretion so that the plasma triacylglycerol level decreases (14).

In perfused rat liver, different fatty acids were taken up at the same rate, but triacylglycerol secretion increased with saturated fatty acid chain length and decreased with the number of double bonds (2). Using a blood-free perfusion medium, Wilcox et al. (14) observed that unsaturated fatty acids promoted a larger triacylglycerol output than saturated fatty acids, but no influence of chain length or number of double bonds within the 2 groups of fatty acids could be found. Also, in cultured hepatocytes, unsaturated fatty acids were more stimulatory than saturated ones (13). Soler-Argilaga et al. (18) found, however, that liver uptake of fatty acids increased with increasing number of double bonds and decreasing chain length. In our system, some differences in uptake were noted but more important was the variability in use of fatty acids for oxidation and esterification, respectively. The differences in esterification, also observed previously (10), were reflected in triacylglycerol secretion (Fig. 2). When the triacylglycerol release was related to cell triacylglycerol radioactivity, it depended on fatty acid chain length but not on unsaturation (Fig. 3). Preliminary analysis by agarose gel chromatography indicated, however, that the lipoprotein particles obtained after incu-

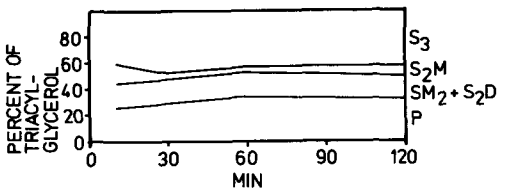

FIG. 4. Distribution of [^{3}H]glycerol among molecular species of triacylglycerol. Isolated hepatocytes were incubated with 1 mM stearate and [^{3}H]-glycerol for different time periods. Cell triacylglycerols were separated into 4 fractions by argentation chromatography. Data are expressed as percent ^{3}H in total triacylglycerol. The unsaturation of the triacylglycerol fractions is designated using the following abbreviations: S, saturated; M, monoenoic; D, dienoic; P, polyenoic.

bation with unsaturated fatty acid were larger than those with saturated ones, supporting data from perfused livers (14).

It can be concluded that the structure of the fatty acid taken up by the liver influences VLDL secretion, although the differences observed in rat model systems vary with experimental setup. So far, it is difficult to decide whether effects on secretion rate or on VLDL composition (14) are most important for the plasma triacylglycerol changes caused by dietary fat modification.

Dietary fat changes may influence lipoprotein triacylglycerol turnover by several other mechanisms, such as increased liver uptake of chylomicron remnants containing saturated fatty acids (19). In addition, a multiplicity of other factors are involved in the regulation of hepatic lipoprotein secretion (20,21).

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METHODS

Horse Erythrocyte Gangliosides: Preparation of the Major Hematoside NeuNG1-Lac-Cer

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ABSTRACT

A simple method for the isolation of hematoside NeuNG1-Lac-Cer from horse erythrocytes is described. An aliquot of the crude ganglioside fraction was labeled by tritiated sodium borohydride after mild periodate oxidation. The compounds obtained were used as radioactive tracers in column chromatography. Gangliosides were applied onto a silicic acid column and eluted stepwise by solvents of steadily increasing polarity. The major ganglioside, NeuNG1-Lac-Cer, was eluted in a high yield by the solvent mixture chloroform/methanol/water (60:35:8, v/v/v).

INTRODUCTION

The hematoside containing *N*-glycolylneuraminic acid (NeuNG1-Lac-Cer) was found to be the major ganglioside from horse erythrocyte membranes (1), although a 4-*O*-acetyl derivative has been identified by Hakomori and Saito (2).

Several chromatographic methods were reported for the preparative isolation of gangliosides more polar than hematosides. Although a specific method for the isolation of liver hematoside has been developed by Seyfried et al. (8), it needed 3 chromatographic systems to obtain a highly purified ganglioside, free of neutral glycolipids, phospholipids and sulfatides. This paper describes a simple chromatographic procedure for the isolation of the horse erythrocyte hematoside using labeled compounds as tracers.

MATERIALS AND METHODS

GM_3 was a gift from Dr. G. Rebel (Centre de Neurochimie, Strasbourg, France); NeuNG1-Lac-Cer, 4-*O*-Ac-NeuNG1-Lac-Cer, C_8-aldehyde-NeuNG1-Lac-Cer, C_7-aldehyde-NeuNG1-Lac-Cer, C_7-NeuNG1-Lac-Cer were gifts from Dr. R. Veh (Institut für Anatomie, Universität Bochum, G.F.R.); GM_1 and GM_2 were gifts from Dr. G. Dacremont (Pediatric Clinic, University of Ghent, Belgium).

Erythrocyte Membrane Isolation

Fresh horse blood was obtained from a slaughter house in Orléans. Membranes were prepared by the Tanner and Boxer method (9). After hemolysis, the resulting ghosts were washed with phosphate buffer (5 mM) and centrifuged. The solid pink pellet was discarded and only the white, light precipitate was collected and freeze-dried.

Lipid Extraction

The lipids were extracted from freeze-dried membranes (1 g) with chloroform/methanol (2:1, v/v) (400 ml). The mixture was stirred vigorously at room temperature for 2 hr. Distilled water (0.15 vol) was added and the mixture was stirred 1 hr. The protein pellet was filtered on glass wool and the filtrate was collected (first extract). The pellet was extracted once more with chloroform/methanol (1:2, v/v). This second extract was pooled with the organic phase of the first extract. The total extract was evaporated to dryness, redissolved in chloroform/methanol (2:1, v/v), filtered and partitioned according to the Folch procedure (10) slightly modified by Puro (4). The lower phase was washed 3 times with water/methanol/chloroform (48:47:3, v/v). The upper phases were gathered with the aqueous phase of the first extract, concentrated under reduced pressure, dialyzed for 3 days at 4 C against a large vol of distilled water (with frequent changes) and freeze-dried. This lipid residue was dissolved in the minimum of chloroform and saponified with 0.2 M NaOH in methanol for 1 hr at room temperature. After neutralization with acetic acid (1 M) and dialysis against distilled water, the content was concen-

trated to dryness and the crude ganglioside fraction was precipitated from acetone at -20 C for 12 hr.

An aliquot of the crude ganglioside fraction (ca. 10 mg) was labeled by NaB^3H_4 after mild periodate oxidation according to Veh et al. (11). These labeled compounds were used as radioactive tracers during the chromatographic step.

Column Chromatography

Lipids (50 mg) were dissolved in chloroform (3 ml) applied to a column (40 × 1.5 cm) packed with Silicic Acid G (Merck, 70-230 mesh) in petroleum ether/chloroform (1:1, v/v) and eluted stepwise by solvents of steadily increasing polarity. The eluting solvents were (A) petroleum ether/chloroform (1:1, v/v): 350 ml; (B) chloroform/ethyl acetate (1:1, v/v):200 ml; (C) ethyl acetate/acetone (1:1, v/v):100 ml; (D) acetone:100 ml; (E) chloroform/methanol (95:5, 90:10, 85:15, 80:20 and 70:30, v/v):400 ml each, and (F) chloroform/methanol/water (75:25:3, 65:25:4, 67:29:4.5, 70:34:5 and 60:35:8, v/v):400 ml each. Five-ml fractions were collected (flow rate: 1 ml/min) and 20 μl of each tube were withdrawn for radioactivity measurement.

Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out on Silica Gel G precoated plates (Merck). Solvent systems were: (I) chloroform/petroleum ether/acetic acid (65:3:2, v/v/v); (II) chloroform/methanol/water (60:35:8, v/v/v); (III) chloroform/methanol/0.5 M ammonia (70:35:3, v/v/v); and (IV) *n*-propanol/water (7:3, v/v).

The plates were first run in solvent I in order to detect and remove neutral lipids and chromophores and to ensure a further clear separation of glycolipids. After drying, the plates were usually run in solvent II. All compounds were located by brief exposure to iodine vapors. After complete sublimation of the iodine, plates were sprayed with 0.2% (w/v) resorcinol in H_2SO_4/water (3:1, v/v) and heated for 5 min. Neutral glycolipids and phospholipids gave yellow-brown spots; cholesterol and cholesteryl esters gave red spots; and gangliosides gave violet spots.

Sugar Analysis

Sugar composition was determined by gas liquid chromatography (GLC) with an Aerograph 2100 gas chromatograph equipped with a flame ionization detector, using a glass column (3 m long) packed with 5% OV 210. The temperature was programmed from 110-210 C (2 C/min), with nitrogen as a carrier gas at a flow rate of 8 ml/min. The gangliosides were subjected to acid methanolysis in methanolysis in methanol/HCl (0.5 N) at 80 C for 24 hr. After extraction of fatty acid methyl esters with *n*-hexane, sugars were analyzed as the trifluoroacetate esters of their *O*-methyl-glycosides (12); *meso*-inositol was used as an internal standard.

Sialic Acid Analysis

Gangliosides were hydrolyzed according to Schauer (13) with 0.2 M formic acid at 80 C for 1 hr. The samples were then applied onto a column (0.3 × 3 cm) of Dowex 1 × 2 (200-400 mesh) formiate form. After washing twice with 5 ml water, the column was eluted with 10 ml of 1 M formic acid to obtain free sialic acid.

The eluate was freeze-dried and sialic acid was identified by GLC after conversion to the trimethylsilyl derivatives (14). Analysis was carried out using a capillary glass column packed with 3% OV 17. The temperature was programmed from 160-260 C (4 C/min). Sialic acid was also identified by TLC on cellulose-coated plates with *n*-butanol/*n*-propanol/0.1 N HCl (1:2:1, v/v/v) as solvent.

RESULTS AND DISCUSSION

In a typical experiment, 7.5 g of freeze-dried erythrocyte membranes were obtained from 5 ℓ of fresh equine blood. The content of lipid-bound neuraminic acid was 4.5 μmol/g freeze-dried membrane. The Folch partition of lipids 2 phases succeeded according to Puro (4), i.e., without salt in the aqueous phase. In his report, Puro convincingly demonstrated that the presence of salt (0.1% KCl) in the upper phase during the Folch partition resulted in considerable loss of the less polar gangliosides (such as hematoside) in the chloroform phase. Table I shows the distribution of sialic acid between organic and aqueous phases in the Folch partition. The recovery of monosialogangliosides in the aqueous phase was 78% after 3 partitions. A fourth partition ensures a more complete recovery yielding to 82% of the gangliosides present in the lipid extract. Puro (4) recovered 88% of bovine kidney gangliosides containing more polar components (GM_1, GD_{1a}, GD_{1b}) that partitioned preferentially in the aqueous phase.

To remove salts and low molecular weight substances, we chose dialysis rather than chromatography on Sephadex. This choice agreed with the recent results of Svennerholm

TABLE I

Distribution of Lipid-bound Neuraminic Acid between Organic and Aqueous Phases in the Folch Partition

	Lipid-bound neuraminic acid (mg)		Recovery in the aqueous phase (%)
	Organic phase	Aqueous phase	
Lipid extract in C/M (2:1, v/v)	1.66		
Partition	1.07	0.57	34
First washing	0.57	0.49	46
Second washing	0.32	0.23	40
Third washing	0.20	0.08	25

Freeze-dried membranes (1 g) were extracted as described in Methods. The vol of the lipid extract was 100 ml. The partition was done with 20 ml distilled water. The washings of the organic phase were done with 0.2 vol water/methanol/chloroform (48:47:3, v/v).

and Fredman (15).

Treatment of the lipid extract with methanolic NaOH resulted in hydrolysis of the 4-*O*-Ac-NeuNG1-Lac-Cer *O*-acetyl group and thus increased the amount of NeuNG1-Lac-Cer. The loss of sialylated compounds did not occur upon precipitation from acetone at -20 C, as demonstrated by TLC of the acetone phase.

The periodate oxidation of the gangliosides affected essentially the C_7-C_9 side chain of neuraminic acid. The experimental conditions were optimized by Veh et al. (11) to yield a maximal C_8-aldehyde neuraminic acid analog. The modified gangliosides behaved as untreated samples in TLC (11) and were used as tracers during column chromatography.

Nonspecific labeling by NaB^3H_4 occurred in agreement with the observations of Gahmberg and Hakomori after labeling of human erythrocytes (16). Thus, all lipid compounds were more or less labeled and could be detected.

Figure 1 shows the elution profile of gangliosides. Up to 300 mg of gangliosides may be applied onto the column without any change in the resolution. Chromophores and neutral lipids were eluted with solvents A and B. Peak I also contained traces of a ganglioside G_3 as shown by TLC. The small peaks preceding peak III contained only cholesterol and fatty acids. Thus, solvents C, D, e_1 and e_2 could be avoided in most cases.

Peak III contained, in addition to cholesterol, a ganglioside G_1 and small amounts of 2 species of neutral glycolipids N_1 and N_2 (which migrated as ceramide monohexoside and ceramide dihexoside). Peak IV contained the compounds present in peak III but in reversed proportions (more glycolipids and less cholesterol). In peak V, a ganglioside G_2 was eluted with G_1 and small amounts of N_1, N_2 and G_3. Peak VI gave a main spot corresponding to G_2 and traces of N_2 and G_3. Peak VII contained G_3 as a single compound. This fraction was devoid of contaminants as tested by TLC after iodine exposure and staining with resorcinol, and its purity was estimated at 99% (Fig. 2). The sugar composition of G_3 was Gal:Glc:NeuNG1 = (1.13:0.93:1.00) and it migrated as the reference compound NeuNG1-lactosylceramide in solvents II, III and IV and was identified as such. The recovery was 37.5 mg of Neu-NG1-Lac-Cer from 50 mg of lipid-bound neuraminic acid applied onto the column (i.e., ca. 75%).

The ganglioside G_1 which migrated faster than the NeuNG1-Lac-Cer has not been analyzed but might be a compound of the GM_4 type (sialyl-Gal-Cer) (17,18), although its presence in the equine erythrocyte membranes

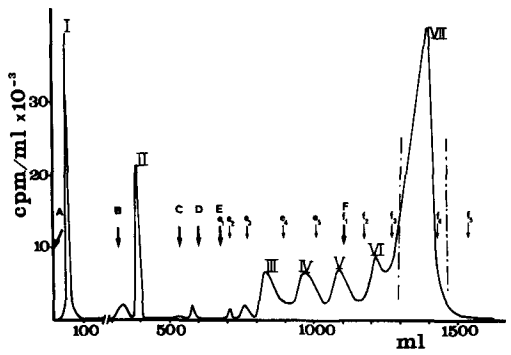

FIG. 1. Elution profile of horse erythrocyte gangliosides on a silicic acid column. Solvents E(e_1, e_2, e_3, e_4, e_5) were chloroform/methanol (95:5, 90:10, 85:15, 80:20, 70:30, v/v, respectively). Solvents F(f_1, f_2, f_3, f_4, f_5) were chloroform/methanol/water (77:25:3, 65:25:4, 67:29:4.5, 70:34:5, 60:35:8, v/v, respectively). The arrows indicate the position of solvent changes.

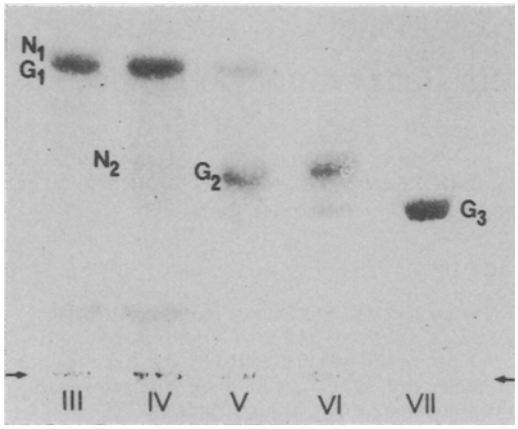

FIG. 2. TLC of the ganglioside fractions eluted with silicic acid column chromatography. The plate was developed with solvent I then solvent II and spots were located with resorcinol reagent.

has not been demonstrated.

The ganglioside G_2 comigrated with 4-*O*-Ac-NeuNG1-Lac-Cer in solvents II, III and IV and was identified as such.

This chromatographic procedure apparently is a suitable method to separate low polar gangliosides from neutral glycolipids without repeated elutions. Radioactive tracers allowed detection of minor components and avoided neuraminic acid assays. This method is particularly adapted to lipid extracts of which the glycolipid pattern is not very intricate, as is the case of horse erythrocyte gangliosides.

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Preparation of Radiolabeled Tetracosa Mono- and Dienoic Acid Methyl Esters from Rat Erythrocyte Lipids by Thin Layer Chromatography[1]

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ABSTRACT

An easy method of obtaining pure fatty acid methyl esters (FAME) of tetracosa mono- and dienoic acids (24:1, 24:2) using thin layer chromatography (TLC) is described. The total lipids isolated from rat erythrocytes were treated with methanolic-NaOH. Sphingomyelin was unaffected by this treatment and was separated from FAME of glycerolipids and cholesterol by TLC. FAME of sphingomyelin were then prepared by acid methanolysis. These esters migrated into 2 distinct bands on TLC. The slow moving band contained FAME of 16:0, 16:1, 18:0, 18:1, 19:0 and 20:0 whereas the fast moving band contained FAME of 22:0, 23:0, 24:0, 24:1 and 24:2. After $AgNO_3$-TLC, the FAME of the fast moving band separated into 3 species; esters of saturated acids, 24:1 and 24:2, respectively. With erythrocyte lipids of rats fed a fat-free diet and injected with ^{14}C-18:1, this method yielded ^{14}C-24:1. From rats injected with ^{14}C-18:2 and maintained on a corn oil diet, ^{14}C-24:2 was obtained.

INTRODUCTION

Fatty acid methyl esters (FAME) prepared from lipids for gas liquid chromatographic (GLC) analysis are generally purified by thin layer chromatography (TLC) (1). We have observed that while the FAME of various glycerolipids migrate as a single band on TLC, those from sphingomyelin separate into 2 bands. Analysis of the composition of the FAME in these bands led us to develop an easy, rapid method for isolating pure methyl esters of tetracosa mono- and dienoic (24:1, 24:2) acids from rat erythrocyte lipids. Furthermore, we used this method to prepare radiolabeled 24:1 and 24:2 FAME from erythrocyte lipids of rats injected with ^{14}C-18:1 and ^{14}C-18:2, respectively.

MATERIALS AND METHODS

In this study, we used pooled total lipid fractions of rat erythrocytes that were prepared from previous investigations (2,3). To a solution of total lipids (0.5 ml) in chloroform/methanol (2:1, v/v), 10 ml of 0.4 N methanolic NaOH was added. After mixing, the solution was kept at room temperature for 45 min. Alkaline methanolysis was stopped by the addition of water (5 ml) and the lipids were extracted with chloroform (6 ml). The extract was washed free of alkali with "Folch upper phase" (chloroform/methanol/water, 3:48:47, v/v) (4).

Sphingomyelin was separated from the FAME, cholesterol and small amounts of glycerophospholipids which were not converted to FAME by TLC using chloroform/methanol/30% methylamine (65:25:8, v/v) (5). If erythrocyte lipids were not treated with methanolic-NaOH and separated by TLC (5), the sphingomyelin fraction was contaminated by small amounts of glycerophospholipids as suggested by the presence of linoleic and arachidonic acids and an appreciably higher level of stearic and oleic acids. Even when other systems were used (6), the isolation of pure sphingomyelin by preparative TLC was difficult because of contamination by phosphatidylcholine (PC) and lysophosphatidylcholine (lyso-PC). Sphingomyelin FAME were prepared by transmethylation using 2% H_2SO_4 in methanol in a N_2 atmosphere in capped culture tubes at 75 C for 16 hr (7). An analysis of the composition of FAME was carried out by the GLC procedures described previously (2). Fatty acid content was determined by GLC using methyl pentadecanoate as an internal standard.

[1-^{14}C]Oleic acid (0.05 mCi; 57 mCi/m mol) and [1-^{14}C]linoleic acid (0.05 mCi; 57 mCi/m mol) were purchased from New England Nuclear, Boston, MA. An aqueous solution of the acids was prepared by mixing an ether solution with 1.15% KCl containing 0.001 M potassium phosphate (pH 7.4) and keeping at 60 C to remove ether.

Two Sprague-Dawley rats (75 g) were obtained from Hilltop Animal Suppliers, Chatsworth, CA, and maintained on a stock diet (Wayne Lab Blox; Allied Mills, Chicago, IL) for 3 days. They were fasted for 18 hr and one rat was injected intraperitoneally with

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[1-^{14}C]oleic acid (0.05 mCi in 1 ml) and fed a fat-free diet (8). The other rat was injected intraperitoneally with ^{14}C linoleic acid (0.05 mCi in 1 ml) and was maintained on a diet containing 15% corn oil (8). After 2 weeks, rats weighing 175 g were anesthetized, exsanguinated and lipids were extracted from their erythrocytes (2).

RESULTS AND DISCUSSION

Sphingomyelin from rat liver (9) or brain (10) has been shown to consist of slow migrating and fast migrating fractions during TLC separation using a developing system containing chloroform, methanol and water. We observed that the sphingomyelin from rat erythrocytes also migrated as 2 distinct bands by TLC using the methylamine system (5). As reported previously, the slow moving sphingomyelin contained acyl groups with 20 and fewer carbons and the fast moving sphingomyelin contained longer acyl groups (9,10).

Fatty acid methyl esters of sphingomyelin also migrated as 2 distinct bands upon TLC separation on Silica Gel H plates with benzene as the developing solvent (Fig. 1). Analysis of the fatty acid composition of the slow moving band showed that it contained esters of 16:0, 16:1, 18:0, 18:1, 19:0 and 20:0 (Fig. 2). The fast moving band contained esters of 22:0, 23:0, 24:0, 24:1 and 24:2. The FAME from the fast moving band were subjected to 10% $AgNO_3$-impregnated silicic acid TLC, using diethyl ether/hexane (20:80, v/v) as the developing solvent (Fig. 3). In this system, FAME separated into 3 bands: esters of saturated acids, 24:1 and 24:2 (Fig. 2). The isolated esters of 24:1 and 24:2 were found to be 99.8 and 99.6% pure, respectively, by GLC (Fig. 3).

Nervonic acid, Δ15 tetracosamonoenoic acid, is biosynthesized by the chain elongation of oleic acid (18:1, Δ9). Tissue levels of oleic acid and its metabolite (20:3, Δ9) increased when rats were fed a linoleate-deficient diet

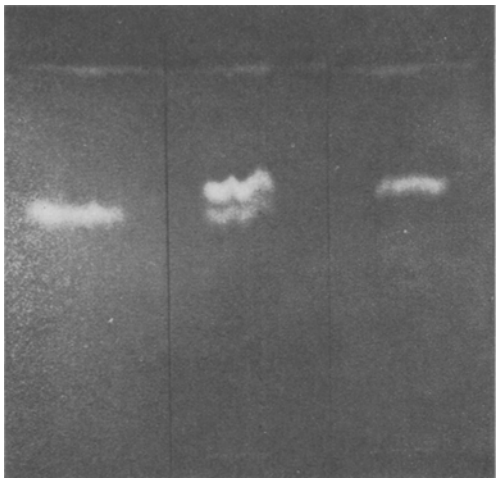

FIG. 1. Separation of fatty acid methyl esters (FAME) of sphingomyelin by TLC. Total FAME of sphingomyelin were separated by TLC on Silica Gel H plates using benzene as the developing solvent. Butylated hydroxytoluene (BHT, 0.05%) was added to benzene as an antioxidant. Methyl esters in the lower, slow-moving (SM) and the upper, fast moving (FM) bands were extracted with chloroform and replated. In the middle lane, total FAME of sphingomyelin were spotted. In the left lane, esters of the SM band and in the right lane those of the FM band, respectively, were spotted.

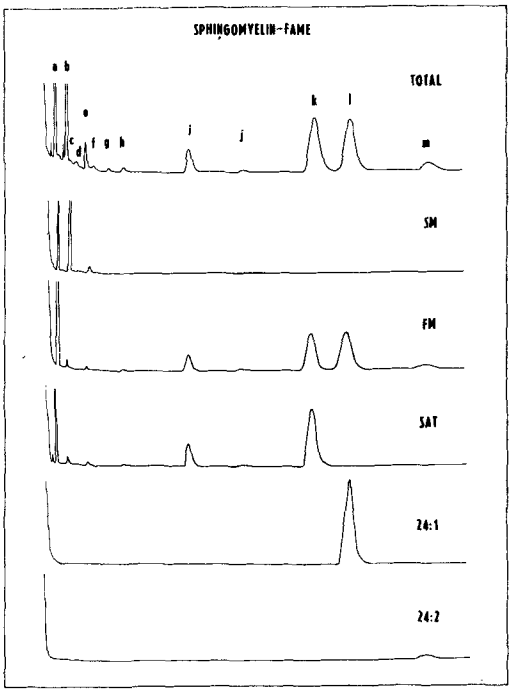

FIG. 2. Separation of fatty acid methyl esters (FAME) of sphingomyelin by GLC. Total FAME of sphingomyelin contained mainly 16:0 (b, 27.3%), 18:0 (e, 2.1%), 20:0 (h, 0.6%), 22:0 (i, 6.9%), 23:0 (j, 0.9%), 24:0 (k, 27.7%), 24:1 (l, 29.5%), 24:2 (m, 3.6%), and trace amounts (less than 0.5%) of 16:1 (c), 17:0 (d), 18:1 (f) and 19:0 (g). BHT eluted as peak a. The FAME of the slow moving (SM) band obtained by the TLC separation (Fig. 1) contained mainly 16:0 (90.3%) and 18:0 (6.2%) and trace levels of 16:1, 17:0, 18:1, 19:0 and 20:0. FAME in the fast moving (FM) band (Fig. 1) contained mainly 22:0 (9.3%), 23:0 (1.6%), 24:0 (36.6%), 24:1 (44.3%), 24:2 (6.8%) and trace levels of 16:1, 18:0 and 20:0. FAME of the FM band were further subjected to $AgNO_3$-impregnated silicic acid TLC (Fig. 3). Esters of the bands corresponding to saturated acids (SAT), 24:1 and 24:2 were analyzed by GLC.

(2,11). Under these conditions, the relative level of 24:1 in the fatty acids of erythrocyte sphingomyelin (31%) was about twice the level of 24:0 (17%) (2). On the other hand, when rats were fed diets containing linoleate, the level of 24:1 in sphingomyelin fatty acids (13%) was about half the level of 24:0 (28%) (2).

Sphingomyelin isolated from tumor cells contains significant levels of 24:2 (12,13). This acid has been identified as Δ15,Δ18 dienoic acid derived from linoleic acid (18:2, Δ9,Δ12). When dogs (14) or rats (2) were fed diets rich in linoleate, the relative level of 24:2 in the fatty acids from erythrocyte sphingomyelin was significantly higher (9-14%) than when they were fed a linoleate-deficient diet (1%). Thus, sphingomyelin rich in 24:1 or 24:2 can be obtained from erythrocytes by maintaining rats on diets deficient in or supplemented with linoleate. Significant amounts of tetracosa mono- and dienoic acids also are present in glycosphingolipids. Analysis of glycosphingolipids from rabbit aorta, plasma and red cells have shown that the relative levels of 24:0, 24:1 and 24:2 are altered by diet (15).

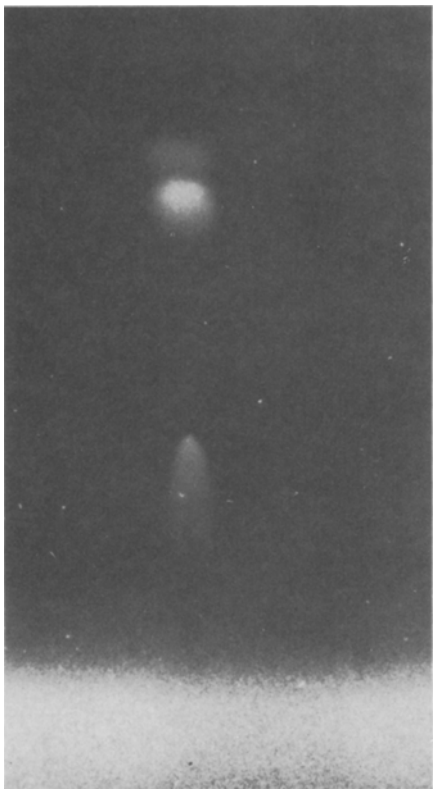

FIG. 3. Separation of FAME from the fast moving band by $AgNO_3$-impregnated silicic acid TLC. The methyl esters were separated on 10% $AgNO_3$-impregnated silica gel plates by using diethyl ether/hexane (20:80, v/v) as the developing solvent. The middle spot contained the methyl ester of 24:1 of 99.8% purity (Fig. 2). The lower spot contained the methyl ester of 24:2 of 99.6% purity (Fig. 2). The upper spot contained FAME of saturated acids (22:0, 17.6%; 23:0, 2.8%; 24:0, 78.4% and trace amounts of 16:0, 18:0 and 20:0) (Fig. 2).

In order to prepare radioactive 24:1, a young rat was injected with ^{14}C-18:1 and to stimulate the synthesis of nervonic acid it was fed a fat-free diet. Similarly, to obtain labeled 24:2, a young rat was injected with ^{14}C-18:2 and maintained on a diet containing 15% corn oil. Since the amount of blood in a rat increases in proportion to its body weight (16), labeled fatty acids will be incorporated for membrane lipid synthesis. We found that the total lipids isolated from erythrocytes of rats given labeled acids were radioactive (1.5 μCi with ^{14}C-18:1 and 0.5 μCi with ^{14}C-18:2). When the TLC procedures just described were applied to the erythrocyte lipids, 0.19 mg of 24:1 (0.14 μCi/μmol) and 0.11 mg 24:2 (0.07 μCi/μmol) were obtained.

The radioactivity observed in 24:1 and 24:2 cannot result from trace contamination by ^{14}C-18:1 and ^{14}C-18:2. This conclusion is based on several experimental observations: (a) sphingomyelin isolated from erythrocytes did not contain any detectable amounts of 18:2 and contained only trace levels of 18:1 (Fig. 2); (b) the FAME of 18:1 and 18:2 migrated slowly and separately from the fast moving band (Fig. 2) that was removed for the separation of 24:1 and 24:2; and (c) we have observed that on TLC with $AgNO_3$-impregnated SiO_2 plates (Fig. 3), methyl esters of 18:1 and 24:1 separated, although both are monoenoic acid derivatives. The amount of radioactivity incorporated in 24:1 and 24:2 was small compared to the initial dose of labeled acids. However, the radiopurity of 24:1 and 24:2, as judged by TLC, was greater than 99%. We also determined the extent of *cis*-isomer present in 24:1 by the TLC procedures described by Hirvisalo and Renkonen (17). No detectable amount of the *trans-isomer* of 24:1 was present. In addition, over 98% of the ^{14}C activity in 24:1 was recovered in the *cis*-isomer. In this study, we have not determined the position of the double bond in 24:1. It is most likely that the tetracosamonoenic acid produced from the labeled oleic acid is nervonic acid, the Δ15 isomer.

Although most fatty acids are components of various lipids, 24:1 and 24:2 are unique in that they are present only in sphingolipids. Hence, the labeled acids prepared can be useful

for studies on sphingolipid synthesis and metabolism. In this study, we have described an easy TLC method to prepare pure 24:1 and 24:2 and demonstrated how this procedure can be adopted to obtain radiolabeled tetracosaenoic acids. The specific activity of 24:1 and 24:2 can be increased by injecting higher doses of radioactive 18:1 and 18:2 into smaller rats.

ACKNOWLEDGMENT

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COMMUNICATIONS

The Δ5 and Δ6 Desaturation of Fatty Acids of Varying Chain Length by Rat Liver: A Preliminary Report

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ABSTRACT

The Δ6 desaturase of rat liver can accommodate substrates with a wide range of chain lengths. Δ9-*cis*,12-*cis*-Dienoic acids of chain lengths 14-22 carbon atoms were all desaturated at the Δ6 position by microsomal preparations from rat liver. By contrast, the Δ5 desaturase appeared much more chain-length sensitive. The percentage Δ5 desaturation of a series of Δ8-*cis*- and Δ9-*trans*-monoenoic acids increased with increasing chain length (from C_{16} to C_{20}).

NOMENCLATURE

Abbreviations found in the text are: ECL, equivalent chain length; DEGS, diethylene glycol succinate; and EFA, essential fatty acid. The nomenclature used to describe fatty acid structure is illustrated by the following examples; palmitic acid, 16:0; oleic acid, 18:1(9*c*); and octadeca-*cis*-9,*trans*-12-dienoic acid, 18:2 (9*c*12*t*).

INTRODUCTION

The chain length specificity of the Δ9 desaturase has been demonstrated using a purified enzyme preparation from rat liver (1). The enzyme has high levels of activity for 16:0 to 19:0 acyl-CoA substrates, and an abrupt cut-off at 20:0. This cut-off probably results from the limiting size of the enzyme "cleft" proposed by Brett et al. (2) which accommodates the acyl chain. The chain length specificities of the Δ5 and Δ6 desaturases have never been examined in a comprehensive study. These desaturases apparently are separate enzymes (3), though each uses acyl-CoA as substrate and NADH and molecular oxygen as cofactors, and is located in the microsomal fraction. Pugh and Kates have recently produced evidence that all-*cis*-8,11,14-eicosatrienoyl phosphatidylcholine may also be directly desaturated at the Δ5 position (4). Since linoleic acid is a good substrate for Δ6 desaturation (3,5), a range of Δ9-*cis*,12-*cis*-dienoic acids was tested as substrates for this reaction. Furthermore, a review of the limited reports in the literature suggested that the C_{16} to C_{20} chain length range would be critical for examination of Δ5 desaturation. As Δ8-*cis*- and Δ9-*trans*-monoenoic acids are known to be Δ5 desaturated (6-8), these C_{16} to C_{20} acids were used in this preliminary study.

MATERIALS AND METHODS

[1-^{14}C] Oleic, linoleic and α-linolenic acids (specific activity 55-60 Ci/mol) were purchased from the Radiochemical Centre, Amersham, U.K. [1-^{14}C] Elaidic and linelaidic acids were prepared by stereomutation of methyl [1-^{14}C]-oleate and linoleate, respectively, using oxides of nitrogen (9). The products were separated by argentation thin layer chromatography (TLC) prior to saponification. All other [1-^{14}C] unsaturated acids were synthesized by chain extension of the appropriate alkyl mesylate with potassium [^{14}C] cyanide (sp act 60 Ci/mol, ex Amersham), followed by methanolysis, argentation TLC and saponification (8). Gas liquid chromatography (GLC) of the methyl esters indicated > 90% mass purity and > 95% radiochemical purity for all substrates. Von Rudloff oxidative cleavage showed no double bond migration (< 1%) had occurred (10).

Details of the enzyme preparation, incubation conditions and product analysis are given in a companion paper (8). Each [1-^{14}C] unsaturated acid (20 nmol) was incubated with unwashed microsomes (8-10 mg protein) from rat liver in the presence of saturating levels of

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cofactors (NADH, NADPH, ATP, CoASH and Mg^{2+} ions). The incubation volume was 2.5 ml, and the incubation was for 1 hr at 37 C, pH 7.3. The percentage desaturation and percentage incorporation into lipid classes are quoted relative to the added [1-^{14}C] acid substrate (100%).

RESULTS AND DISCUSSION

In these preliminary experiments, the substrate concentration was nonsaturating (8 μM), so the percentage desaturation cannot be directly equated with the maximal rate of desaturation. Although substrate saturation is the proper approach for studying enzyme properties, we chose nonsaturating conditions because the higher percentage desaturation of the substrate facilitated product identification.

Δ6 Desaturation

The percentage Δ6 desaturation of a series of C_{14}-C_{22} [1-^{14}C] *cis*-9,*cis*-12-dienoic acids is reported in Table I. Linoleic acid, a natural substrate with a high rate of Δ6 desaturation (5), gave the lowest percentage desaturation. [1-^{14}C] Acyl groups were extensively incorporated into lipids, with only 2-8% of the added radioactivity remaining as the free acid at the end of the incubation. Thus, activation of the novel acids to their acyl-CoA thioesters must be occurring. ECL values for both substrate and product methyl esters on a DEGS GC column also are shown in Table I.

The direct demonstration of Δ6 desaturation of fatty acids of different chain lengths has, until recently, been limited to acids of C_{16}-C_{18} chain length (3,5,11). However, since the initiation of this study, Castuma et al. (12) have demonstrated the Δ6 desaturation of 20:2(9*c*, 12*c*) and 20:3(9*c*12*c*15*c*) in vitro, although the rates for these C_{20} substrates were lower than for 18:2(9*c*12*c*) and 18:3(9*c*12*c*15*c*), respectively. Schlenk et al. noted that 19:4(7*c*10*c*13*c* 16*c*) fed to rats deficient in EFA produced 21:5(6*c*9*c*12*c*15*c*18*c*) (13), whereas Bridges and Coniglio have reported the biosynthesis of 24:4(9*c*12*c*15*c*18*c*) and 24:5(6*c*9*c*12*c*15*c*18*c*) from arachidonate in rat testes (14). However, these results do not prove that the Δ6 desaturase is capable of acting on longer chain polyenoic acids ($\geqslant C_{20}$), as 2 routes are possible. The Δ6-*cis*-polyenoic acids could result from chain elongation of existing Δ7-*cis*-polyenoic acids, followed by Δ6 desaturation, or from chain elongation of Δ4-*cis*-polyenoic acids, which are the products of a hypothetical Δ4 desaturation step (3,15).

The results shown in Table I clearly demonstrate that the Δ6 desaturase can accommodate a wide range of substrate chain lengths. Unlike the Δ9 desaturase (1,2), there is no abrupt cut-off in activity for chain lengths longer than the natural substrate chain length (C_{18}), indicating that the substrate binding-site is open-ended. Also, a shortening of the methyl end of the substrate is not critical to Δ6 desaturation. A full study is now required in order to determine the effect of chain length on desaturation velocity and apparent K_m. An interesting point to note is that, according to this investigation with nonsaturating substrate concentrations,

TABLE I

Δ6 Desaturation of Fatty Acids of Varying Chain Length by Rat Liver Microsomes

Substrate	ECL values[a]		Δ6 Desaturation (%)[b]		^{14}C-Incorporation (%) into:	
	Substrate	Product			Neutral lipids[c]	Polar lipids[d]
14:2(9*c*12*c*)	16.5	17.1	18.5	(24)	33	59
16:2(9*c*12*c*)	17.7	18.3	24	(35)	16	79
18:2(9*c*12*c*)	19.4	19.95	16	(13)	17	76
20:2(9*c*12*c*)	21.1	21.7	32.5	(37.5)	39	54
22:2(9*c*12*c*)	23.0	23.6	75.5	(74)	58	34
18:1(9*c*)	18.6	19.2	4	(5)	17	79
18:3(9*c*12*c*15*c*)	20.4	20.95	33	(46.5)	12	86

[a]Packed 10% DEGS column, 180 C.

[b]Details of the incubations are in ref. 8. In a separate experiment, the substrate acids were added complexed to defatted bovine serum albumin (2.5 mg BSA/ml of incubation medium). The Δ6 desaturations for this experiment are reported in parentheses.

[c]Principally triacylglycerols, but also diacylglycerols.

[d]Includes phosphatidylcholine, phosphatidylethanolamine and small amounts of acyl-CoA.

20:2(9*c*12*c*) is a better substrate than 18:2(9*c* 12*c*), whereas maximal velocity measurements indicate the converse is true (12). The important question then arises as to which in vitro situation best represents in vivo metabolism. This is an open question.

Δ5 Desaturation

Table II shows the percentage Δ5 desaturation of some novel acids and also 2 natural substrates, 20:2(11*c*14*c*) and 20:3(8*c*11*c*14*c*). The results are for a single microsomal preparation when the entire range of novel acids was tested. Several additional experiments were performed using parts of the series, but as similar levels of Δ5 desaturation were observed, these are not reported. The C_{20} all-*cis*-polyenoic acids had the highest percentage Δ5 desaturations. With the 2 series of monoenoic acids, there was a distinct chain length bias. The [1-^{14}C] acyl groups were extensively incorporated into lipids, with only 2-10% of the added label remaining as free acid after 1 hr. ECL values for both substrate and product methyl esters on a DEGS GC column are also reported in Table II. The difficulty with the detection and measurement of low levels of Δ5 desaturation of Δ9-*trans*-acids using a packed, polar GC column, because of the unusually small differential in ECL value between Δ9-*trans*- and Δ5-*cis*,9-*trans*-acids, has already been noted (8). Since this study was completed, Mahfouz and Holman have reported the Δ5 desaturation of 18:1(8*c*) (6). Also, the Δ5 desaturation of 18:1 (9*t*) agrees with the observations of Lemarchal and Bornens (7).

Examination of the sparse body of data available on Δ5 desaturation reveals a substrate specificity with a probable chain length limitation. In vitro studies show higher rates of Δ5 desaturation for C_{20} polyunsaturated acids over C_{18} polyunsaturated acids (5,16). Experiments performed in vivo also support the hypothesis that the C_{20} chain length is preferred over shorter chain lengths. Sprecher and Lee have shown that C_{19} to C_{21} *cis*-11,*cis*-14-dienoic acids are all desaturated at the Δ5 position, with the greatest conversion in vivo occurring for the C_{20} acid (17). Δ5 Desaturation of 20:1(11*c*) was low, whereas none was observed for 18:1(11*c*) (17,18). Feeding 17:2(8*c*11*c*) to rats deficient in EFA did not result in the production of trienoic acids (19). In summary, a chain length specificity of $C_{17} < C_{18} < C_{19} < C_{20}$ seems likely for Δ5 desaturation. Furthermore, the Schlenk et al. studies suggest that 23:5(8*c*11*c*14*c*17*c*20*c*) and 22:4(8*c*11*c*14*c*17*c*) are good substrates for Δ5 desaturation (13). The results from Table II show that, as chain length decreases from C_{20} to C_{16} for the series of 8-*cis*- and 9-*trans*-monoenoic acids tested, Δ5 desaturation falls to a barely detectable level. These preliminary results lend credence to the hypothesis that Δ5 desaturation is strongly under chain-length control, and the authors hope that this will stimulate the complete study now required.

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TABLE II

Δ5 Desaturation of Fatty Acids of Varying Chain Length by Rat Liver Microsomes

Substrate	ECL values[a]		Δ5 Desaturation (%)[b]	^{14}C-Incorporation (%) into:	
	Substrate	Product		Neutral lipids[c]	Polar lipids[d]
20:2(11*c*14*c*)	21.25	21.75	59	18	72
20:3(8*c*11*c*14*c*)	21.75	22.2	60.5	9	89
16:1(8*c*)	16.8	17.4	1	22	72
18:1(8*c*)	18.55	19.1	7	27	64
20:1(8*c*)	20.4	20.9	18.5	37	56
17:1(9*t*)	17.65	18.05	3	6	84
18:1(9*t*)	18.6	19.0	7.5	11	87
20:1(9*t*)	20.4	20.8	55	45	53
18:2(9*t*12*t*)	19.35	19.75	5	22	76

[a]Packed 10% DEGS column, 180 C.

[b]Details of the incubations are in ref. 8.

[c]Principally triacylglycerols, but also diacylglycerols.

[d]Includes phosphatidylcholine, phosphatidylethanolamine and small amounts of acyl-CoA.

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Dinosterol in Model Membranes: Fluorescence Polarization Studies

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ABSTRACT

The interaction between the phospholipids of *Crypthecodinium cohnii*, a heterotropic marine dinoflagellate, and its major sterol dinosterol was studied using a fluorescence polarization technique. Compared to cholesterol, dinosterol is less soluble in model membranes and as effective in increasing the microviscosity. These results indicate that the unique side chain of dinosterol does not play a special role in terms of complementary interaction with the phospholipids of this organism.

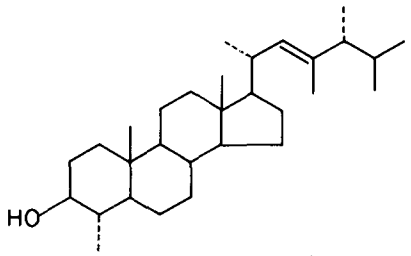
INTRODUCTION

Marine sterols contain complex side chains which are not found in terrestrial organisms. Previously (1,2) we discussed the possible functional significance of the unusual structure of various marine sterols. We hypothesized that a special stabilization of the membranes of the organism may be gained by a complementary structural interaction between the sterol and other membrane lipids.

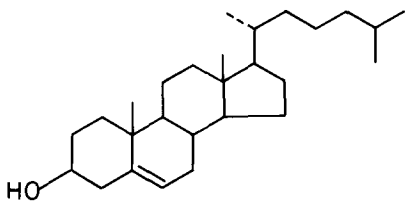
Dinosterol (I) was selected for various reasons (Fig. 1). The unprecedented pattern of alkylation of its side chain is of special importance from a biosynthetic point of view. It occurs in high proportions (ca. 30-60% of the total sterol mixture) in various species of marine dinoflagellates (3,4). The most characteristic polyunsaturated fatty acid of marine dinoflagellates is docosahexaenoic acid (22:6) (5). In *Crypthecodinium cohnii*, an easily grown heterotropic marine dinoflagellate, the major polar lipid fraction, phosphatidylcholine (PC), was found to contain up to 66% 22:6 (6), whereas dinosterol constituted 48% of the sterol mixture (3). This association in vivo of a highly polyunsaturated phospholipid with unusually high proportions of dinosterol (I) might indicate the existence of a mutual hydrophobic affinity. This prompted us to study the interaction of dinosterol with synthetic PC (16:0, 22:6) and with the total phospholipid mixture of *C. cohnii* in model membranes compared to the interaction of cholesterol (II) using fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe. Lala et al. (7) have demonstrated that the microviscosity of the membrane, which is derived from the polarization, is sensitive to structural changes in the sterol backbone.

MATERIALS AND METHODS

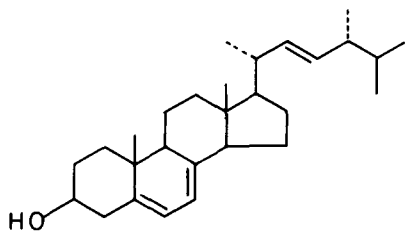
C. cohnii (Woods Hole strain d) was grown in La Jolla, CA, at 28 C in 60-ℓ (4 15-ℓ bottles) of MLH medium (8) and harvested at stationary phase of growth after 14 days. Cells (yield, 66 g wet weight; ca. 17 g dry weight) were quickly frozen on Dry Ice prior to extraction and quantitative analysis of phospholipids (for qualitative evaluation see ref. 9).



DINOSTEROL (I)



CHOLESTEROL (II)



ERGOSTEROL (III)

FIG. 1. Structures of dinosterol, cholesterol and ergosterol.

Preparation of Membranes

We preferred not to use sonication to form membranes because of the high susceptibility of the docosahexaenoate chain to oxidation. The ethanol injection method (10), which causes the formation of single-bilayered vesicles, was used to prepare PC (16:0,22:6) and egg PC membranes with or without sterols. The amount of sterol incorporated into the membrane was determined, after centrifugation and extraction, by gas liquid chromatography (GLC). Multilamellar liposomes were prepared from the phospholipid mixture of *C. cohnii* for fluorescence studies because of the mixture's insolubility in ethanol using a procedure reported (11) to produce uniform preparations with constant polarization values.

The fluidity of the membranes was estimated by the fluorescence polarization technique with DPH as a fluorescence probe (12). Measurements were made at 25 C on a SPEX Fluorolog spectrofluorimeter operated in the E/R mode. The exitation and emission wavelengths were 365 nm and 428 nm, respectively, with respective bandwidths of 5 nm and 10 nm. Results are expressed as polarization of fluorescence, an increased value indicating a reduced rate of probe motion and by inference a more restrictive hydrophobic environment (13). Approximate values of microviscosity ($\bar{n}$) were evaluated according to the expression derived by Shinitzky and Barenholz (14).

RESULTS AND DISCUSSION

Composition of *C. cohnii* Phospholipids

The phospholipid mixture of *C. cohnii* (62% of the polar lipids mixture) was shown by thin layer chromatography (TLC) and phosphorus analysis (15) to consist of PC (71%), lysophosphatidylcholine (lyso-PC, 8%), phosphatidylethanolamine (PE, 8%), phosphatidylinositol and phosphatidylserine (7%), and cardiolipin (6%). The fatty acid composition (% mol) of the phospholipid mixture was as follows: 14:0(15%), 16:0(25%), 18:0(2%), 18:1(14%) and 22:6(44%).

Incorporation of Dinosterol into PC Vesicles

Attempts to form membranes from egg PC or PC (16:0,22:6) with sterol concentration higher than 15% mol resulted in clear and turbid solutions for cholesterol and dinosterol, respectively. The same results were obtained when sonication was applied. GLC determination of dinosterol in the membrane also gave a maximal value of 15% mol. Attempts to incorporate dinosterol to a higher extent in multilamellar liposome prepared from egg PC, PC (16:0,22:6) or *C. cohnii* phospholipids mixture were unsuccessful.

Fluorescence Polarization

Table I shows the effect of cholesterol (II) and dinosterol (I) (15% mol) on the polarization and microviscosity of different membrane preparations. The results do not distinguish between the effect of the 2 sterols (in 15% mol) on the fluidity of the membranes. Of the 3 kinds of membranes, egg PC is the most sensitive to the addition of sterol.

The ability of sterol to be incorporated into liposomes of egg PC depends on its structure. Demel et al. (16) have found that the solubility limit of ergosterol (III) in egg PC liposomes is 25% mol (50% mol for cholesterol). This is probably the result of the steric hindrance caused by the bulky side chain of the sterol. It was also shown (17) that the ability of ergos-

TABLE I

Effect of Cholesterol and Dinosterol on the Polarization and Microviscosity of Various Membrane Preparations

Membrane	Polarization[a]	Microviscosity (poise)
Egg PC[b]	0.138	0.86
Egg PC/cholesterol	0.177	1.26
Egg PC/dinosterol	0.177	1.26
PC(16:0,22:6)	0.111	0.64
PC(16:0,22:6)/cholesterol	0.138	0.86
PC(16:0,22:6)/dinosterol	0.138	0.86
C. cohnii phospholipids	0.116	0.67
C. cohnii phospholipids/cholesterol	0.134	0.82
C. cohnii phospholipids/dinosterol	0.127	0.76

[a]Deviations between duplicates did not exceed 5%.

[b]PC = phosphatidylcholine.

terol to order egg PC membranes is equal to that of cholesterol only up to 15-20%. Compared to ergosterol, the hydrocarbon tail of dinosterol is more bulky, thus decreasing the sterol solubility. This behavior might be explained if one assumes that formation of sterol dimer or sterol-sterol interaction is taking place at a concentration above 20% mol (18). Such interaction might be favored for cholesterol but unfavored for ergosterol (III) and dinosterol (II). Probably this is a dominant factor because membranes prepared from PC (16:0, 22:6) or *C. cohnii* phospholipids did not accommodate more dinosterol than egg PC membrane. Possible "solubilizers" for dinosterol in *C. cohnii* membranes might be membrane proteins. For example, high affinity of the Folch-Lees protein for cholesterol was demonstrated by monolayer experiments (19). As the results have shown, the increase in the viscosity of egg PC membrane, upon addition of cholesterol or dinosterol, is higher than that of PC (16:0,22:6). Similar results were obtained by Demel et al. (20). Lala et al. (7) have shown that the membrane microviscosity is sensitive to methylation of the sterol nucleus at C_3, C_4 or C_{14} and that 4α-methylcholestanol (personal communication) condenses the membrane nearly as well as cholesterol. Therefore, it is not surprising that the 4α-methyl group of dinosterol does not notably affect its membrane condensation property, compared to cholesterol.

At this time, we conclude that a special kind of structural complementarity between the phospholipids and the major sterol of *C. cohnii* is not detectable by the fluorescence polarization technique. The functional role of dinosterol still needs to be elucidated.

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Cholesterol Methodology for Human Studies

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ABSTRACT

A classification and review of the methodology involved in the determination of serum cholesterol for human (or animal) studies are presented. The purpose of both is to enable selection of a technique appropriate for the assay intended with a reasonable understanding of its advantages, disadvantages and limitations. The various methods discussed include direct reaction systems, partial isolation systems and complete isolation systems, as well as screening, reference and definitive procedures. The interferences that could occur are considered, especially those caused by hemoglobin, the turbidity in lipidemia, and bilirubin, as well as interferences caused by optical aberrations and chemical reactants. The various instrumental methods used to determine cholesterol or a substitute determinand such as hydrogen peroxide are discussed, including spectrophotometry, electrochemistry and densitometry of electrophoretically separated proteins.

INTRODUCTION

The beginnings of modern cholesterol methodology date from 1885-1909 (1-3), when color-forming and precipitation reactions for cholesterol were developed, of which modifications are still used for the determination of serum cholesterol. The keys to the technology were the Liebermann-Burchard (LB) color reaction (1,2) and the digitonin precipitation reaction (3) for cholesterol, encompassing a combination of principles and ideas that enabled procedures to be evolved for the determination of free, esterified and total cholesterol. With relatively little alteration, but with some analytical honing, these are still in use. A novel development of the early fifties involved attempts to determine cholesterol in serum directly without a prior separatory purification step (4,5). Although there was some resistance to such apparently bold technology—a resistance which waxed and waned—the idea of direct determination not only persisted, but apparently extended to other serum determinands (the constituent determined, also analyte). It was demanded by most of the simplified robots, the large devices with automation characteristics precluding, for the most part, the preparation of extracts and filtrates or other more complicated separatory steps.

While the LB reaction was enjoying peak use, the cholesterol oxidase reaction was discovered (6,7) and some preliminary procedures were suggested (8,9), although none of them were suitable for routine use. An important development was the appearance of a relatively simple and less cumbersome technique employing cholesterol oxidase (cholesterol: oxygen oxido-reductase, EC 1.1.3.7) in a process in which the hydrogen peroxide generated was used in a peroxidase (EC 1.11.1.7)-coupled reaction to generate a colored product (10). The next critical addition involved the inclusion of cholesterol esterase (EC 2.1.1.13) (11) in the procedure which eliminated the need for the alkaline saponification of cholesteryl esters, which did not react with the oxidase. Introduction of 4-aminoantipyrene and phenol from the Trinder reaction for glucose (12) into the peroxidase-coupled reaction for determining the hydrogen peroxide resulted in a complete procedure. From this point, the optimization of matrix characteristics, along with the substitution of other reagents for 4-aminoantipyrene and phenol, contributed to the overall improvement of a single-pipetting, one-step analytical technique (13,14). A modified procedure employed catalase to convert the hydrogen peroxide and methanol to formaldehyde. Then, by coupling the formaldehyde with acetylacetone and ammonia, the Hantzsch reaction (15) was used for the determination of the peroxide. Thus far, this modification has not been used as commonly as the Trinder reaction.

This discussion will focus on current technology in a manner that will assist the reader in selecting from a wide array of available methods. An all-inclusive classification will be suggested, built on well received published reviews (16-20). However, it will not attempt arrangement of methods on the basis of the final color reactions, thereby avoiding the problems engendered by classifying them as functions of terminal equilibrium reactions (16). Instead, it will group methods according to the manner of sample treatment that ultimately leads to the quantitative reaction selected, because, after all, the final step of a methodological scheme is not the method. Obviously, direct determinations may be complex, even though the procedure only calls for the addition of serum to the reagents. This

single-pipetting step may initiate a series of sequential chemical reactions, terminating in an equilibrium reaction of a color formation, which is used to make the final measurement.

METHODS

Direct Reaction

In this class of methods, no separation or partial separation of the analytical phases occurs. Obviously, when all constituents of the sample are present and it is simply mixed with reagents, maximal perturbance by potential interferences may be expected. These effects could depend on the instrumental methods used to determine the equilibrium product, such as photometry or electrochemical analysis. Generally, spectrophotometry is used because that is how most determinands are assayed in the clinical laboratory. Electrochemical analysis is or can also be used for the determination of cholesterol, but this is still relatively rare.

Blank determinations are probably necessary. However, often the only blank included is the reagent blank, which may be inadequate if sources of irrelevant absorption are present in the sample. The most common natural interferences encountered in serum are bilirubin, hemoglobin and the turbidity in hyperlipidemia. Sometimes, only one interfering substance is present, but encountering 2 or more is a distinct possibility. The use of a serum blank in which one component of the reagent may be left out will sometimes be feasible, provided the substance remains spectrally identical both in the serum blank solution and in the final reaction system, for then an absolute correction can be made. But if the substance to be blanked is a static reactor in the blank and a dynamic reactor in the specimen, serum blanking may be difficult (21). Sometimes the interfering material has a complex reactive nature and can form more than one kind of reaction product, as can be true for bilirubin. It has been shown that it may then generate different irrelevant absorptions in the sample blank and in the sample (22). In that case, the correction for a variable dynamic interference may be erroneous (23).

The main advantage of the direct approach is, of course, that the procedure is simple because the sample is merely added to the reagents; this simplicity also facilitates automation or mechanization, especially with robots which can handle only 1- or 2- part reagents at best. The major disadvantage of the direct procedure is that, since no phase separation such as extraction or dialysis occurs, maximal interference effects not only are possible, but prevalent. In the case of absolute or constant errors from these interferences, little correction may be required. In the case of proportional (relative) errors, the principle of standard additions may be used (24). If the interfering material is an interactor enhancing or inhibiting the reaction, then the standard addition technique, by means of taking advantage of this interacting process, will allow correct values to be closely approximated, even though incorrect absorbance signals are obtained.

Attempts to overcome the problem of interference in direct procedures by correcting at some wavelength other than the measuring wavelength have been made using bichromatic spectrophotometry (25). Several of the automatic instruments now use dual wavelength measurements for most of their determinations in biological fluids (e.g., Hycel M., Abbott ABA-100, 200 and VP, and TECHNICON STAC), whereas other instruments use dual wavelength measurements only where it is believed to be essential (e.g., Dupont ACA). However, it is obviously difficult to assure that one side-wavelength absorbance used for any interference that one might encounter in a biological specimen will subtractively correct the peak-wavelength absorbance for that interference. In addition, the choice of the 2 wavelengths is severely limited by the abridged character of the filter photometers in the automatic instruments. In order to make such a correction accurately, dual wavelength monochromators capable of narrow waveband selection are preferable; the nature of the interference should be known and no other interference can be tolerated at either wavelength, unless it gives identical readings at both wavelengths. Such severe restrictions would make the dual wavelength approach difficult when the limited wavelength monochromators of present instruments are used, unless there is little or no interference present.

In direct methods where enzyme reagents are used for a final colorimetric reaction, free and total cholesterol determinations are possible, depending on whether cholesterol esterase is present in the reagents.

Partial Purification — Use of Organic Solvents

In this class of methods a partial sample cleanup is effected primarily by a separatory process, involving extraction from the proteins with water-soluble solvents (26) or liquid-liquid extractions of the cholesterol from the proteins into organic solvents (27). In the liquid-liquid extractions, this transfer is aided by having a water-soluble component in the extraction mixture, e.g., chloroform-methanol. Free and esterified cholesterol are determined in the

extract using digitonin, which precipitates free cholesterol and separates it from its esters.

The main requirement for blank determinations after partial purification is the reagent blank. Many interfering materials are removed during the extraction process. Digitonin precipitation also aids in separating the cholesterol from most but not all interfering substances. The main drawback with digitonin precipitation is that other 3-β hydroxy sterols are precipitated by the saponin along with cholesterol, although their molar absorptivities in either the LB or the ferric chloride reactions may be considerably lower than that of cholesterol.

An advantage of partial purification is obviously that the equilibrium reaction mixture is purer, as many, though not all, of the interferences are eliminated (28). However, the potential for automation decreases as the complexity of a procedure increases.

In the early matrices used for the LB reaction, esterified cholesterol produced considerably more color than did free cholesterol, which was a significant disadvantage of partial purification. Therefore, procedures for total cholesterol, involving alkaline saponification followed by extraction from the alkali, were developed which virtually eliminated LB-reactive compounds other than cholesterol from the equilibrium reaction (28). This method became the reference by which other proposed procedures are judged even today (18).

A novel approach to partial purification involves electrophoresis of serum in cellulose acetate, filter paper or a transparent gel such as agarose (29-32). Several of these techniques have been proposed as a means of separating the carriers of cholesterol, the lipoproteins. They are visualized by overlaying them with enzyme reagents, which results in a color complex involving the hydrogen peroxide-peroxidase oxidative coupling action of 4-aminoantipyrene and phenol. This procedure has several theoretical advantages over chemical fractionation of serum by selective precipitation of several of the lipoproteins. It is also simpler to carry out than ultracentrifugation followed by the determination of cholesterol in the separated fractions.

Complete Isolation of Cholesterol

The first nearly successful attempt to isolate cholesterol completely involved extraction, then saponification, to convert esters to free cholesterol followed by saponin precipitation with digitonin (3) or tomatine (33) and washing of the insoluble digitonide or tomatide. The dried precipitate was then dissolved and determined by the LB equilibrium reaction (34). This process also obviated the problem that esterified cholesterol gives a color that is ca. 17% more intense than that of free cholesterol, (35) as an apparent matrix phenomenon (18). The procedure is designated "nearly successful" because the extraction and precipitation steps cannot ensure the absolute purification of the determinand.

A number of chromatographic systems involving thin layers, filter paper and columns were elaborated which give more complete isolation than saponin precipitation (18). Gas chromatography with various detectors (36,37), including the mass spectrometer (38), has been applied to the determination of cholesterol in serum.

Screening, Reference and Definitive Procedures

Screening large populations for their serum cholesterol concentrations has been simplified by the invention of automation. This mechanized approach to rapid performance of a large number of determinations has made it possible to examine, without great expenditure of labor, all incoming patients in hospitals as well as outpatients.

The advent of rather accurate cholesterol procedures based on enzyme reagents has not only improved the screening capabilities, but also increased the reliability of the analysis with a concomitant reduction of the doubtful range above and below the normal range. The mechanical simplicity of direct enzymatic reagent techniques has enabled automation to be applied to a large number of clinical samples. The accuracy approaches that of the reference procedures and the normal or reference range can now be more reliably defined.

Thus far, no attempt to use enzyme reagents in the final step of a reference procedure has been published. The procedures accepted as reference procedures still terminate in the LB reaction, i.e., the Abell et al. (28) and Schoenheimer-Sperry (34) procedures. However, since the reference procedure on which these 2 techniques are based is the gravimetric Windaus procedure (3), it should also be considered a reference procedure even though it is unlikely that it would be performed now.

There is no agreement on definitive procedures (18), but likely candidates include gas chromatography with a mass spectrometer as detector or some version of high performance liquid chromatograph. Undoubtedly, the results obtained with well-designed procedures terminating in enzyme reactions will closely approximate the definitive values and easily match the current reference values.

DISCUSSION

Interferences

The naturally occurring interfering materials commonly encountered in serum specimens are bilirubin, hemoglobin and the turbidity in severe lipemia. A variety or sample blanks are described for direct procedures in which an attempt is made to correct for a static or dynamic interference encountered. Two kinds of blanks commonly used are prepared in the following way. The blank reagent is made up with a missing reactive component. This omission can either allow a static interference to represent the total background of irrelevant absorbance or it can permit a dynamic interference to react while the determinand, cholesterol, is unreactive. Dual wavelength blanks are used to make a correction at some wavelength at which the interference is assumed to be identical to that at the peak wavelength of the cholesterol reaction or to apply the proportion of interference at some wavelength to correct the peak wavelength (25).

Since most modern clinical methods are mechanized and limited in the complexity of sample handling by the automated systems, there has been a tendency to use direct handling and thus interference has been maximized. Of the 3 interferences mentioned, the simplest to correct is that resulting from hemoglobin, because its color effect is easily eliminated using a serum blank. Such a treatment, incidentally, is possible only with a genuinely unreactive interfering substance, i.e., a static interference.

Bilirubin presents a much more formidable problem for the analyst (39). It reacts independent of the cholesterol reaction in strong acid to form stable biliverdin. It also interacts in the final step of the peroxidase-coupled enzyme reagent systems and competes with the 4-aminoantipyrene-phenol as a substrate of that reaction. Fortunately, in this instance, the residual bilirubin and perhaps its oxidized product almost completely substitute for the intended oxidation product derived from 4-aminoantipyrene and phenol. The result is a useful compensating error. But, if one now subtracts a serum blank, as some suggest, the final result will be too low (40). However, in the case of colorless drugs which could act similar to bilirubin in the peroxidase-coupled reaction, the final results would be too low with or without a serum blank, a fact already demonstrated for the virtually identical glucose oxidase reaction (41). In this instance, no compensatory color would be present to counteract the loss of color from the peroxidase substrate. It is easy to correct for excess color resulting from the generation of biliverdin from bilirubin if the bilirubin concentration is known. This is ca. 5 mg/mg bilirubin for the LB reaction and ca. 0.7 mg/mg for the ferric chloride reaction. One reason for the difference in magnitude of error for these 2 acidic systems is that the LB reaction for cholesterol produces a peak at the wavelength where biliverdin has a spectral peak, whereas biliverdin shows a minimum in its absorption spectrum at the absorption maximum of the ferric chloride reaction. Another reason is inherent in the molar absorptivities of the 2 acidic reactions for cholesterol. The ferric chloride reaction for cholesterol is much more sensitive than the LB reaction.

The problem in severe lipemia is more complicated than suspected at first glance. In direct reactions of the strongly acidic systems at high dilutions, the turbidity may clear up as a result of the dilution and the nature of the medium. However, at the nearly neutral pH of the enzyme reagent systems severely lipemic specimens may cause turbidity in the final solution, which may require correction, e.g., by a serum blank. If one attempts to clarify the serum by ultracentrifugation in the presently popular micro-ultracentrifuges sold particularly for that purpose, some other factors may have to be considered. One factor involves the lipids in the chylomicrons that are centrifuged off. They contain a small quantity of cholesterol, but perhaps it is small enough to be tolerable as a negative error. However, the removal of a very high concentration of chylomicrons could decrease the volume of serum enough to concentrate the remaining cholesterol by as much as 20-30% (42). This phenomenon has been a problem in other assay systems (43).

Enhancement and inhibition of the ferric chloride reaction for cholesterol by bromide and thiouracil, respectively, have been described when they are present in serum. These interacting species produce proportional errors. Unlike independent side reactions, which cause absolute errors, proportional errors are correctable by the method of standard additions (24).

Electrochemical methods for the determination of cholesterol are relatively new (44-46). They are based on the measurement by the oxygen electrode of the rate of oxygen used up during the cholesterol oxidase reaction or on the measurement by a peroxide electrode of the rate of formation of the peroxide generated in the same reaction. Any side reaction involving either oxygen or peroxide could, in theory, affect the reaction. So far, the oxygen electrode technique has been free of such criticism, although one report suggests that it is not as precise as it could be (47).

Standardization

A previous review of cholesterol methodology provides a rather complete discussion of the problems of standardization of the several procedures commonly used (18). There are differences in reactivity between free and esterified cholesterol in LB reactions which vary as a function of the differences in matrices. There is also the dilemma of what to use as a standard for direct reactions in automated instrumental determinations. If one uses the values for a standard serum the concentration of which has been determined by a reference procedure, there may be considerable background absorbance in the samples to be analyzed, which may be quite different from that of the standard.

The temperature of reaction can disrupt LB procedures if the standard is prepared in a solvent such as glacial acetic acid, which generates little heat upon mixing with LB reagent, whereas serum with its high water content generates relatively much more heat for a temperature-dependent reaction (23).

In enzyme reagent systems, 3 alternatives are currently available. In one, nonesterified cholesterol is dissolved in isopropanol, and this water-soluble solvent generates a molar absorptivity similar to the one obtained with the cholesterol of the sample (48). In order to avoid the use of organic solvents entirely, aqueous standards have been prepared using a detergent to solubilize nonesterified cholesterol (49,50). This standard can be used for the strong acid systems and for the enzyme reagent systems. Morpholine cholesterol hemisuccinate is a practical cholesterol standard in aqueous solution, as shown for the ferric chloride and LB reactions (51). When cholesterol is determined in a multiphasic analyzer, a serum secondary standard is necessary, because each sample is subjected to a variety of tests simultaneously. For this, previously assayed pooled human serum is required and the background problems described earlier in this discussion must be considered (18).

Equilibrium Reactions

A variety of equilibrium reactions and some kinetic reactions have been described in the course of evolution of the determination of cholesterol (18). These have mostly involved reagents which react with cholesterol to generate a measureable product, usually a pigment. Sometimes, the reagent is determined along with the cholesterol e.g., by weighing an insoluble complex. The gravimetric method was actually the first successful approach to the determination of cholesterol (3). Digitonin quantitatively reacts with the free hydroxyl group of the A ring to form an insoluble complex which, when washed free of impurities, provides an accurate weighing form of cholesterol. Some years later, reactions with the carbohydrate moiety of the pentasaccharide precipitating agent provided an indirect determination of cholesterol by assaying for the concentration of saponin involved in the precipitation (52,53). The LB reaction, which was invented some years earlier, provides a simpler photometric solution to the problem. The reagent, a mixture of acetic anhydride, acetic acid and sulfuric acid, produces green products with both free and esterified cholesterol or with the cholesterol digitonide. The LB reaction has undergone many modifications through the years and is still widely used either in the form of the sodium sulfate-stabilized reagent of Huang et al. (54) or the *p*-toluene sulfonic acid technique of Pearson et al. (5).

In 1953, another color reagent was proposed for generating pigment by a direct reaction with cholesterol. It is a mixture of ferric choride in an acetic acid-sulfuric acid milieu, which produces a stable purple compound with considerably higher molar absorptivity than the one produced by the LB reaction. It has also generated several modifications, including ferrous sulfate (55), ferric perchlorate (56) and a mixture of ferric acetate-uranium acetate and ferrous sulfate (57). The direct reaction with serum was replaced by a partial isolation procedure (58), but then reverted to the direct reaction (56). All of these are still used, some manually and some in automated procedures. Interferences, including reagent contamination, e.g., nitrite in sulfuric acid (59) and drug interference, e.g., bromide and thiouracil, have been encountered (60,61). The presence of other steroids in tissue determinations has been described, leading to claims for the advantages of one procedure (57) over others (52-64). However, in a direct comparison of the equilibrium reactions themselves, where constant concentrations of interfering material were used, these claims have been determined to be unfounded (60).

Additional spectrophotometric reactions for the peroxidase-coupled step are certain to be proposed in the future, and if they are more sensitive, they should be useful for the determination of low cholesterol concentrations, e.g., in serum fractions with HDL cholesterol or in cerebrospinal fluid (65). They could be based on fluorescence (66), chemiluminescence (67), or color reactions (68,69). An increase in sensitivity would also be useful for kinetic

studies, where only a portion of the signal of the equilibrium reaction is used (70).

Reaction Mechanisms

The reaction mechanisms of the enzyme reagents are well understood for both the Trinder and Hantzsch reactions (18). Here, oxygen is used and cholstenone is formed along with hydrogen peroxide. The procedures based on reagent oxygen or either of the 2 reaction products, hydrogen peroxide or cholestenone, clearly show that the mechanism is correct. The mechanisms of the LB and iron reaction are more obscure because the products are difficult to identify or isolate. In a rigorous proof of mechanism (71), it was shown that both reactions begin with the dehydration to a common carbonium ion, but then follow separate oxidative pathways. The LB reaction proceeds to a measurable but unstable green pentaenylic ion, which can be oxidized to a yellow cholestahexaene sulfonic acid, a product which has also been a measuring form. The iron reaction is described as proceeding oxidatively through a dienylic carbonium ion to a trienylic ion and finally to a stable purple tetraenylic ion, the compound which is measured.

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Analysis and Physiologic Significance of Cholesterol Epoxide in Animal Tissues

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ABSTRACT

The presence of 5α,6α-epoxy-cholestan-3β-ol, an oxidation product of cholesterol, has been demonstrated in a variety of animal tissues. No definitive biologic role for this sterol has been forthcoming, although the physiologic implications of its occurrence are many. The presence of the epoxide in ultraviolet (UV)-irradiated skin was detected by a combination of thin layer chromatography (TLC) and gas liquid radiochromatographic (radio-GLC) techniques in 1971 and led to the suggestion that it might be responsible for the carcinogenic properties of UV. Subsequently, in vivo levels of this sterol in the skin of UV-irradiated animals were quantitated by radio-GLC after TLC separation and preparation of the radiolabeled acetate ester. Presence of the compound in mouse liver was detected by GLC of the trimethylsilyl (TMS) ether and confirmed by mass spectrometry (MS). In all cases, however, in vivo quantitations were complicated by substrate induction of sterol epoxide hydrase, an enzyme that hydrates the epoxide to form cholestan-3β,5α,6β-triol (triol). Consequently, the relationship of the sterol epoxide to UV-carcinogenesis is as yet unclear and recent studies suggest that if this sterol is involved, then further metabolism of the compound must be required. A practical means of examining the metabolites of the epoxide was developed, employing radio-TLC scanning. Using this procedure, the relationships of substrate concentration, time of reaction and pH optimum to reaction rate were determined. Although in short term in vitro incubations of liver homogenates the bulk of epoxide is hydrated to triol, several other metabolites are detectable by TLC autoradiography. Even though the case for direct involvement of cholesterol epoxide in the etiology of carcinogenesis or other diseases is equivocal, the epoxide or its metabolites may yet prove valuable as a diagnostic aid in revealing abnormal function associated with certain diseases.

INTRODUCTION

Prior to the late 1960s, it was generally believed that, except for a few toad venoms, the occurrence of steroid epoxides was relatively rare in nature (1). Although minor sterols of the cholestane series had been isolated from various biological materials, little notice was taken as these could have easily represented artifacts from the autoxidation of the parent cholesterol molecule. In 1971, however, 2 reports appeared in which cholesterol epoxide was identified in biological samples (2,3). Both reports piqued interest as to the potential physiologic role(s) this compound plays. Although Kadis (4) recently has reviewed the role of various steroid epoxides in biologic systems, it is the intention here to focus only on cholesterol epoxide, its analysis and metabolism, with respect to its physiologic potential.

METHODS OF ANALYSIS

Gray et al. (2) isolated and identified cholesterol epoxide from abnormal human sera. After extraction with chloroform/methanol (2:1), the serum lipid extract was subjected to preparative thin layer chromatography (TLC) on Silica Gel G. Sterols, after hydrolysis of the sterol ester fraction, were fractionated either as the trimethylsilyl (TMS) ethers or in free form on silver nitrate-impregnated silica gel thin layers. Further analysis of the epoxide was achieved by gas liquid chromatography (GLC) using columns packed with either 1% OV-1 or OV-17. Identity was verified by mass spectrometry (MS). Fioriti and Sims (5) had earlier used TLC and GLC methods to identify cholesterol epoxide in a mixture of autoxidation products of cholesterol. However, Gray and coworkers provided several arguments why the epoxide they had identified could not have resulted from autoxidation of serum cholesterol.

Cholesterol epoxide was also isolated from ultraviolet (UV) light-irradiated human skin which had been preincubated with radiolabeled cholesterol (3,6). Total lipids were extracted with chloroform/methanol and preliminary separation of the lipid classes was obtained by TLC on Silicar-7GF plates. Cholesterol-derived photo-products were observed as polar materials remaining at the origin of the chromatograms and verified by silicic acid column chromatography. Tentative identification of cholesterol epoxide in the radioactive skin extract was obtained by cochromatography with unlabeled reference standards. A band corresponding to the R_f of the reference epoxide was eluted and subjected to radio-GLC analysis in which the effluent from the split

stream was collected in glass cartridges packed with 3% DC-200 silicone fluid on Florisil, and counted in a liquid scintillation counter (7,8).

These observations of the photo-induced formation of cholesterol epoxide were weakened by their dependence on the uptake of radiolabeled parent compound. It is well known that many radiolabeled organic compounds are more prone to autoxidation. Thus, proof of the origin of epoxide in UV-irradiated mice was only obtained when skin extracts were processed in the absence of radiolabeled cholesterol and the corresponding TLC-resolved fractions were esterified with radiolabeled acetic anhydride and subjected to radio-GLC analysis (9). In chronically irradiated animals, ca. 16 μg of cholesterol epoxide/g skin, compared to 0-2 μg for nonirradiated controls, occurred after 10 weeks.

Cholesterol epoxide was also identified in the liver of the hairless mouse (10). Lipid extracts were first fractionated by TLC in 1,2-dichloroethane. The polar materials remaining at the origin were eluted and rechromatographed in chloroform/acetone (9:1). The area corresponding to the R_f of authentic epoxide was eluted and some samples were used directly for MS. Other samples were esterified with radiolabeled acetic anhydride, and the acetate esters were analyzed by radio-GLC. Still other samples were derivatized with *bis*-trimethylsilylacetamide and the TMS ethers were analyzed by GLC on a 10-ft × 2 mm id column packed with 3% OV-1. An approximate level of 80 ng of epoxide/g liver was present. Special precautions during isolation, as well as control experiments, precluded the possibility that the low levels of epoxide isolated from liver were artifacts of sample preparation.

METABOLISM

Fioriti et al. (11) fed rats diets containing 1.5% cholesterol epoxide for 90 days and examined several tissues for the presence of the sterol. No trace of the epoxide was found in any of the tissues examined, but about 50% of the ingested epoxide could be accounted for in the fecal lipids. In subsequent studies, it was demonstrated that with time after intubation, an inverse relationship occurred between epoxide level in the gastrointestinal (GI) tract and an unknown sterol. The unknown compound was identified as cholestan-3β,5α,6β-triol. The triol is known to evoke a number of physiologic responses (12-14).

Martin and Nicholas (15) reported that radiolabeled cholesteryl palmitate, when incubated with the microsomal fraction of adult rat brain fortified with a NADPH-generating system, formed a mixture of cholesterol-5,6-epoxides. Of the total radioactivity eluted from the cholesterol epoxide region after TLC, ca. 60% cocrystallized with the α-epoxide isomer. Free cholesterol incubated under similar conditions did not yield epoxides. Longer incubations resulted in triol formation.

Mitton et al. (16), in a study of cholesterol oxidation by a rat liver microsomal preparation, found cholestan-3β,5α,6β-triol among the oxidation products. They suggested that the triol could have arisen from cholesterol epoxides; when the radiolabeled α-epoxide isomer was supplied as substrate, the triol was one of the products isolated. Aringer and Eneroth (17) also demonstrated the formation of cholesterol-5,6-epoxides in rat liver microsomal fractions. These workers found that the epoxides were formed under conditions favoring autoxidation and suggested their occurrence could be related to lipid peroxidase activity. In addition, they found that the β-epoxide was formed in 3-4-fold excess over its α-isomer. In this regard, it is notable that Smith and Kulig (18) have proposed that cholesterol epoxides in tissues may derive from an initial oxidation of cholesterol by molecular oxygen to form cholesterol hydroperoxides which then subsequently attack the parent molecule. Under such laboratory conditions, they obtained ratios of α-:β-isomers of 1:8 to 1:11, respectively. Recently, Sevanian et al. (19) reported that cholesterol epoxides were formed in rat lungs exposed to oxidizing gases. They, too, proposed that lipid autoxidation may contribute to the levels of epoxide formed. However, using the same methods of analysis, they found vastly different ratios of the epoxides from that predicted by Smith and Kulig (18), i.e., 8:1 for the α and β isomers, respectively. Ratios of 1:1 were obtained after BF_3 methanolysis and separation by TLC. These discrepancies emphasize the importance of adequate analytical methods. The recent application of high performance liquid chromatographic (HPLC) techniques by Tsai et al. (20) for the separation of the cholesterol epoxide isomers may benefit future studies of relative epoxide content in biological samples.

Chan and Black (21) examined the distribution of cholesterol epoxide in UV-irradiated mouse skin. Highest levels of the epoxide were found in the epidermis and were distributed rather evenly throughout all subcellular epidermal fractions. Potentially significant levels of the compound, however, were capable of diffusing from UV-irradiated epidermis, suggesting ready access to systemic involvement. In

this regard, Bowden et al. (22) found that when radiolabeled cholesterol epoxide was painted on the skin of mice, or administered by gastric intubation, it was rapidly and primarily excreted in the feces. Only residual amounts of the unaltered epoxide remained at the site of application and, with time, a portion of the radiolabeled materials recovered from the GI tract was present as water-soluble conjugates. These workers found very low levels of epoxide metabolites at the site of application even 72 hr after multiple administration of the compound for 4 days. Compatible with this observation is the finding that cholesterol epoxide hydrase activity was not increased in rat lung tissues at a time after NO_2 exposure when epoxide levels were elevated (19). It was surmised that cholesterol epoxide may not be an effective hydrase activator and that levels of the epoxide, and thus tissue residence time, would remain unchecked under conditions which led to epoxide formation. However, cholesterol epoxide hydrase activity was 96% greater in skin of hairless mice receiving suberythemic UV irradiation for 15 weeks, when compared to nonirradiated controls (23). It was concluded that the hydrase enzyme was indeed substrate-inducible and responsible for the concomitant reduction in skin epoxide levels which occurred prior to tumor onset. A probable explanation for the divergent conclusions concerning substrate inducibility of the hydrase lies in the lengthy response time between first appearance of substrate and detectable increases of the enzyme. Alternatively, tissues of different origin undoubtedly possess different enzymatic capacity and may have restricted biosynthetic capability in response to the substrate. Certainly, mouse liver has an inherently greater capacity for epoxide hydration than does skin and this quality may account for the low levels of epoxide found in the liver. Although the epoxide may not be an efficient hydrase inducer, at least in skin the relationship between substrate and enzyme activity seems evident; the enzyme activity is responsible for modulation of epoxide levels over a prolonged time period.

A practical radiochromatographic assay for cholesterol epoxide hydrase has been described (24). When hydrase activity in mouse liver homogenates was examined using this method, ca. 70% of the radioactivity was recovered as triol after 30 min incubation—a time during which the reaction approached linearity. About 10% of the radiolabel occurred as unidentified metabolites. The pH optimum was shown to be 7.4. An apparent K_m of 1.49 μM and a V_{max} of 0.452 μg/mg protein/hr was determined for a substrate concentration which approached saturation level and yet provided sufficient conversion of substrate to be accurately quantitated.

PHYSIOLOGIC ROLE

The occurrence of cholesterol epoxide in a number of animal tissues under varied anomalous or experimental conditions indicates the potential and putative roles associated with this compound. The finding by Gray et al. (2) that cholesterol epoxide was present in sera of patients with varying degrees of hypercholesterolemia and atherosclerosis, but not in normal volunteers, suggested a relationship between severity of atherosclerosis and serum epoxide levels. As yet, definitive evidence for this important thesis has not been forthcoming. These investigators failed to find triol in human sera and suggested that the epoxide was not metabolized in man in a similar manner to that reported for rodents. After intubation of rats with cholesterol epoxide, Fioriti et al. (11,25) did not find the compound in the blood. Only residual levels were recovered in mice blood 18 hr after gastric intubation (22). Recently, however, triol has been identified in human feces. The triol resulted from microbial metabolism of epoxide and its occurrence indicated that hydrase activity is present in the human colon (26). These observations indicate not only a similarity between epoxide metabolism in man and rodents, but imply that ingested cholesterol epoxide is metabolized in a very different manner from that which might occur endogenously in the blood. Thus, elevated serum epoxide levels may be a manifestation of abnormal sterol metabolism and represent a rather specific marker of atherosclerotic disease. This aspect certainly deserves further investigation.

Several oxygenated sterols, including cholesterol epoxide, have been associated with the semeiology of Wolman's disease (27). Cholesterol epoxide esters were isolated from tissues of Wolman's disease victims and it was speculated that the hydrolysis of 7-α-hydroxycholesteryl esters was impaired, thus leading to the accumulation of this oxygenated esterified sterol; this was presumed to act as a precursor for the formation of cholesterol epoxide esters. In this case, the occurrence of cholesterol epoxide appears as a manifestation of physiologic dysfunction rather than as an active participant in the etiology of the disease.

Oxygenated sterols also are known to produce adverse effects in several in vitro systems. Chen et al. (28,29) have demonstrated

growth inhibition of cells in culture by oxygenated sterols and have theorized that the observed restriction of sterol synthesis by these compounds results in altered cell membrane fluidity, permeability and activities of membrane-localized enzymes. Cytotoxic effects of cholesterol epoxide on cells in culture also have been reported (30,31). In addition, oxygenated photo-products of cholesterol, including the epoxide, have been shown to inhibit lipogenesis in human skin slices (32).

Cholesterol epoxide also has been implicated in the carcinogenic process. It was Roffo (33) who first hypothesized that photo-induced oxidation products of cholesterol may be responsible for the carcinogenic effects of UV-radiation. Although no carcinogenic hydrocarbons could be identified in photo-oxidized cholesterol preparations (34), Fieser (35) suggested that, if the carcinogenic compound were a steroid, it could have escaped detection. Massive testing efforts of cholesterol-derived oxidation products were undertaken by Bischoff and coworkers (36,37). These investigators reported that cholesterol epoxide, when injected subcutaneously, induced sarcomas in both rats and mice. Seelkopf and Salfelder (38), however, observed no carcinogenic effect when the compound was administered in the diet of rats and mice.

Black and coworkers (7,9,39) have examined the potential role of cholesterol epoxide in UV-carcinogenesis. Although increased levels of the epoxide occurred in the skin of chronically irradiated mice just prior to the appearance of tumors, no direct carcinogenic effect by the compound has been demonstrated. Antioxidants, known to impede lipid peroxidation, were shown to inhibit the immediate photochemical conversion of skin cholesterol to its epoxide (40). Furthermore, dietary antioxidants are effective in suppressing the formation of UV-induced tumors (41,42). However, when epoxide levels were determined for chronically irradiated animals receiving antioxidant-supplemented diets and in which tumor formation was inhibited, the concentrations were higher than in control animals on unsupplemented diets and with a higher tumor incidence (39). It is apparent that cholesterol epoxide could not be the ultimate carcinogen responsible for UV-induced skin cancer. But, the possibility that the epoxide acts as a precarcinogen requiring further metabolic activation to a carcinogenic agent was not ruled out.

Moreover, Reddy et al. (43) have postulated that cholesterol epoxide may be of etiological significance in colon carcinogenesis. Its principal metabolite, triol, was found in increased levels in feces of patients with ulcerative colitis and colon cancer (44). However, when instilled intrarectally in rats, the epoxide and triol both failed to produce tumors or to enhance N-methyl-N'-nitro-N-nitrosoguanidine-initiated carcinogenesis.

The conflicting and fugacious nature of the evidence for cholesterol epoxide's role in carcinogenesis in vivo is paralled by in vitro systems as well. The compound has been reported to lack mutagenic activity, direct and metabolically induced, in the Ames microbial assay (45,46, cf. 4). On the other hand, Parsons and Goss (47) have demonstrated that the epoxide induces a similar degree of chromosome damage and DNA repair synthesis in human fibroblasts as do low doses of UV radiation. Despite this carcinogen-like effect, they were unable to induce transformation of the cell line with the epoxide and speculated that further studies of the sterol's mode of action might be difficult because it failed to cause detectable DNA breaks or other DNA damage. However, Kelsey and Pienta (48) have demonstrated that the epoxide was able to transform hamster embryo cells. Previous studies with the aid of their system had shown a good correlation with in vivo carcinogenicity for a broad spectrum of chemical compounds.

Although no definitive evidence has yet been developed to indict cholesterol epoxide in the etiology of any disease, the circumstances suggest that if it is not directly involved in a causal manner in major diseases, such as atherosclerosis or certain types of cancer, it may yet be useful as a diagnostic aid in detecting these disease states, and its physiologic potential deserves further study.

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The Analysis of Urinary Hormonal Steroids

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ABSTRACT

A survey is given of current trends in the assay of urinary hormonal steroids. Both group assay methodology and assays for single urinary steroids are reviewed as are semi-automated and automated procedures and high-resolution and high-capacity techniques, as applied to the profile analysis of urinary steroid hormones.

INTRODUCTION

It is half a century since the isolation of crystalline estrone from urine by Doisy and Butenandt in 1929, of estradiol by Marrian and Doisy in 1930 and of androsterone by Butenandt in 1931. Ever since, the steroids excreted in this biological fluid have continued to supply valuable information about the metabolism of steroid hormones in man and animals.

The relative value of this information has declined as sophisticated new techniques have enabled the determination of steroids in ever smaller quantities in endocrine organs, blood and body tissues, and in subcellular structures. But the very advances in technological sophistication that have expanded the areas of inquiry have also increased the potential of the methodology currently available for the study of urinary hormonal steroids. This short survey will focus on some recent developments in the field of hormonal urinary analysis with no attempt to cover the area in a systematic or exhaustive manner.

FROM BIOLOGICAL ASSAY VIA GROUP ASSAYS TO THE ESTIMATION OF INDIVIDUAL HORMONAL STEROIDS IN URINE

The road to current analytical methodology for the estimation of urinary steroids has been an arduous and long one and is perhaps best illustrated by following the assay, e.g., for neutral 17-oxosteroids in urine from biological assays to current methodology for individual neutral 17-oxosteroids. In the 1930s, the assay of choice for androgens in urine was the capon's comb biological assay, as used by, among others, Gallagher and Koch (1) and McCullagh and Cuyler (2). This was a complicated assay requiring capons some 6 months after castration and a 3-5-day experimental period—obviously very expensive and time-consuming. The description by Zimmermann in 1935 (3) of a chemical reaction in which *m*-dinitrobenzene gives a purple color with neutral 17-oxosteroids led, within a decade, to a gradual switch away from biological assays to group assays for neutral 17-oxosteroids in urine, based on the Zimmermann reaction. Although it was realized that the information obtained with the chemical assay was in many cases somewhat different from that inferred from the biological assays, the savings in time and cost were so considerable that the bioassays simply went out of use. Instrumental in this development was the establishment of micro methods (4,5), requiring only a few milliliters of urine for an assay of reasonable reproducibility. Many changes and improvements have been suggested for the routine assay of 17-oxosteroids in urine over the years. An interesting development was an attempt by a Medical Research Committee on Clinical Endocrinology in Great Britain to standardize the methodology for the group assay of 17-oxosteroids (6). While in some ways this might be beneficial to some laboratories, it seems questionable in principle, since this would tend to stifle innovation and further development of new methodology.

The next major step in the assay of neutral 17-oxosteroids as a group was the development of semi-automated and almost automated methodology for this type of assay based both on the continuous flow principle and on batch operation, as reviewed previously (7). Such methodology is faster than manual methods and results are more consistent.

The discrepancies in results between bioassay and group assays of neutral 17-oxosteroids obtained at times led early to attempts to establish assay methods for individual neutral 17-oxosteroids in urine. Some of the fundamental work in this area was done by Dingemanse et al. (8) and by Dobriner et al. (9) with liquid chromatography. Later, other forms for chromatography were used extensively in the isolation of individual urinary neutral 17-oxosteroids, as reviewed by Bush (10) and by Heftmann (11). Particularly important in the last few years have been the development of capillary column gas chromatography and high-resolution liquid chromatography. Important for large-scale analyses of urinary

17-oxosteroids have been the semi-automated assays of individual neutral 17-oxosteroids, as reviewed elsewhere (7,12). Competitive protein binding assays, which have risen to a dominant position in many areas of steroid methodology in recent years, have not been used much for urinary neutral 17-oxosteroids, perhaps because of the well-established chromatographic techniques that so efficiently cover the profile analysis of these compounds.

The development in methodology for neutral 17-oxosteroids in urine is prototypical for the general development in the assay of urinary steroid hormones, with the exception of the very few competitive protein binding assays. In the analysis of other urinary steroids, the development has also been from bioassays—now practically abandoned for the assay of hormonal steroids in urine—via group assays to the current methodology for individual steroids. Certain group assays survive in many laboratories, but unquestionably the tendency is toward specific methods for individual urinary steroids, and the move towards automation with computer interface can be expected to progress steadily.

Group Assays for Urinary Steroid Hormones

Group assays have been developed not only for the 17-oxosteroids but also for neutral corticosteroids and estrogens—all steroids of importance in medicine (13).

Group assays for neutral corticosteroids. Two general types of group assays for corticosteroids have been established over the years. One methodology is based on the reaction between phenylhydrazine in concentrated sulfuric acid and steroids containing the dihydroxy acetone side chain (14) and the other on the determination of the 17-oxosteroids formed by oxidation with bismuthate (15) or periodate (16) after initial reduction of the 17-oxosteroids with sodium borohydride. As has been the case for the 17-oxosteroid assay, many modifications of the 2 types of assays have been published over the years.

Group assays for total estrogens. Among the many assays suggested for the determination of total estrogens in urine, one of the most widely used has been the rapid method described by Brown et al. (17). It uses a semi-automatic extractor and colorimetry or spectrofluorometry by the Kober-Ittrich method, in which the phenolic group of the estrogen molecule reacts with a mixture of phenol and sulfuric acid and the product is then extracted by an organic solvent containing *p*-nitrophenol.

Semi-automated ana automated analyses for the group assay of hormonal steroids in urine. There has been increasing emphasis on automation in many areas of analytical chemistry, and automated and semi-automated techniques for the group assay of steroids in urine have been proposed for all the major groups of steroids.

17-Oxosteroids. Semi-automated methods for total 17-oxosteroids were introduced by Zak and Epstein (18) and Zak et al. (19), based on the continuous-flow principle. Ganis et al. (20,21) devised an amyl acetate extraction procedure in their continuous-flow methodology, and Egloff et al. (22) analyzed a urine blank along with the urine sample to compensate for unspecific background. Vestergaard and Bachman (23) have developed a semi-automated batch analysis system with computer calculation of the data and spectrophotometry at 3 different wavelengths.

17-Oxogenic steroids. Zak et al. (19) have adapted their 17-oxosteroid method to the assay of 17-oxogenic steroids and Caisey and Child (24), as well as Laue (25) have developed semi-automated assays for oxogenic steroids in which a solvent exchanger is used in a continuous-flow system after periodate oxidation of urines. A semi-automated continuous-flow method based on the Porter-Silber reaction and having a capacity of 10 analyses/hr has been introduced by Sparagana et al. (26). We have (13) modified our semi-automated-17-oxogenic steroid methodology (27) to serve as a high-capacity batch analysis method for 17-oxogenic steroids.

Estrogens. A number of methods have been described for the semi-automated and automated estimation of estrogens in urine. They have generally been based on continuous-flow methodology. Examples are the Hainsworth and Hall (28) method, which employs the Kober-Ittrich fluorescence reaction after extraction by an organic solvent, and the colorimetric semi-automated assay described by Yee and Jackson (29), in which the reaction of estrogens with 3-methyl-2-benzothiazolinone hydrazone is used.

Advantages of automated and semi-automated assays. Many more analyses can be performed per day with automation than with manual methods. With these methods, from 10 to 80 analyses can be carried out per hr and they generally provide better reproducibility. They are obviously best suited for laboratories having heavy work loads of urinary analyses, but they may be too costly for laboratories performing only occasional group assays for hormonal steroids.

Caution in the use of group assays. One

dilemma in the use of group assays and of many of the more specific assays for individual steroids is the selection of the hydrolysis procedure necessary to free the steroids from the conjugate forms in which they are excreted in the urine. Some methods have been suggested which circumvent this dilemma by a direct assay of the conjugated steroids without hydrolysis. However, practically all generally used methods employ an initial hydrolysis step based on either chemical hydrolysis or on the use of appropriate enzyme preparations. Acid hydrolysis, as commonly used in routine group assays of urinary steroids, leads to some loss and a good deal of artifact formation; the use of enzyme preparations poses its own problems, e.g., enzyme inhibitors in the urine may give underestimates of the steroids present.

Drug intake has been found to interfere with all the common group assays for urinary steroids in varying degrees. In the 17-oxosteroid assay, e.g., many drugs can interfere with the determination, as reviewed elsewhere (30). Similarly, some drugs interfere with the Porter-Silber and 17-oxogenic group assays for corticosteroids (31), and several drugs may lead to erroneous assays based on the Kober reaction (32).

The specificity of the group assays has been established reasonably well for the most commonly used methods through experience gathered over decades, including a comparison of the sums of individual steroids assayed chromatographically with group assays. Such comparisons have been performed by Vestergaard (33) for 17-oxosteroids and 17-oxogenic steroids and by Brown et al. (17) for the estrogens. However, this does not mean that, e.g., assays developed for human urine can uncritically be applied to other animal species.

The intelligent use of group assays in medicine presupposes an acquaintance with many factors—endogenous and exogenous, physiological and pathological—that may influence these values, e.g., those recently reviewed for the neutral hormonal steroids in the urine (34).

Assays for Single Urinary Steroid Hormones

The direct assay of single hormonal steroids in urine demands methodology of high selectivity and high sensitivity. In a few exceptional cases, large amounts of a steroid may be excreted and in such a situation a direct estimation by simple physicochemical means may be possible. Otherwise, initial separation steps—and that currently means, in most cases, chromatography in one form or another—is necessary if methodology not using protein-binding steps is employed. The development of protein-binding methodology has opened the way for direct nonchromatographic estimations of steroids in urine because of the combination of high sensitivity and selectivity possible in this type of assay.

Methods Other than Protein-binding Assays

Direct estimations. The only urinary analysis for a single steroid not involving initial purification or chromatography that has been used extensively is the Venning method (35,36), now only of historical interest, in which conjugated pregnanediol was estimated after precipitation and weighing of the precipitate.

Estimation after chromatography. Many methods of this nature have been devised, and some of them are still in use in various laboratories. Among the better known and more extensively used assays has been the Klopper et al. (37) method for pregnanediol, involving chromatography on alumina, acetylation and colorimetry based on a modified sulfuric acid reaction. The Fotherby and Love pregnanetriol method (38) based on chromatography on alumina and a sulfuric acid color reaction is another example taken from this group. Urinary aldosterone has been determined by chromatography and by the use of color reactions or fluorescence using many different analytical approaches, as surveyed by Gottfried et al. (39). Testosterone has been estimated after chromatography by a micro adaptation of the Zimmermann reaction (40) or by fluorometry (41). Cortisol methods involving fluorometry have been proposed by, e.g., De Moor et al. (42), but these are probably not entirely specific for cortisol.

Chromatography and double-isotope dilution methods. This is now an infrequently used, complex and cumbersome assay methodology involving several chromatographic steps and the formation of doubly labeled derivatives. A representative example is the double-isotope dilution assay for testosterone described by Gandy and Peterson (43), but similar methods have also been developed for many other urinary steroids.

Gas chromatography. This technique is currently in wide use, particularly when more than one hormonal steroid is to be estimated. Examples of gas chromatography used for the estimation of single urinary hormonal steroids are the methods for testosterone (44,45), pregnanetriol (46), pregnanediol (47), estrone and estradiol (48), aldosterone (49) and tetrahydroaldosterone (50).

Gas chromatography/mass spectrometry. Powerful and useful techniques have evolved

from the interfacing of the gas chromatograph with the mass spectrometer. The specificity obtainable by this combined technique is unsurpassed in the field of steroid analysis, and the procedures developed by combining the high resolution of gas chromatography with the high specificity of the mass spectrometer have become reference methods in many areas of biochemical analysis.

This combined technique has been used both for profile analyses and for the estimation of single steroids in urine. The best methodology in this area uses as the internal standard stable, isotope-labeled steroids with an increase in m/e numbers of 2 or more and selective ion monitoring at appropriate m/e numbers (mass fragmentography). Mass fragmentographic methods have been described for estradiol (51), estriol (52), testosterone (53), aldosterone (54), estratetrol (55) and progesterone (56). Field-desorption mass spectrometry has been used in the analysis of a steroid conjugate, estriol 16α-glucuronide by Aldercreutz et al. (57).

Protein-binding assays for single urinary steroid hormones. The 1970s has been the decade of protein-binding assays in the field of steroid hormone analysis. Assays based on both binding to carrier proteins and radioimmune techniques have flourished. The end of the decade saw the appearance of practical assays, based on the use of enzymes rather than isotopes as markers in the immunoassays. The principle of the competitive protein-binding assays has been described in a number of monographs (58-60). Survey chapters on the assay of steroids by this methodology are presented in these publications and a recent review has been published by Pratt (61). By far, the majority of assays were primarily developed for the determination of single steroids in plasma, but for some urinary steroids of particular importance in medicine, methods have also been devised. Early in the development of protein-binding analysis, the antibodies used for the assay generally showed considerable cross-reactivity with related compounds, and chromatographic separation or other purification steps were often required before performing the competitive binding reaction. Advances in the production methods for antibodies now allow direct estimation without preliminary chromatography in some assays with considerable savings in time and effort. Another innovation is the direct estimation of steroid conjugates by eliminating the time-consuming hydrolysis.

Protein-binding assays for urinary steroid hormones, either free or released by hydrolysis. One frequently used assay in this area has been the competitive protein-binding assay for urinary cortisol developed by Murphy (62). This is a carrier-protein assay, based on competition between sample cortisol and tritiated cortisol for binding sites on the corticosteroid-binding globulin of human plasma. It does not involve chromatography. Radioimmunoassays for cortisol have also been established, e.g., by Ruder et al. (63). More than 12 radioimmunoassays for aldosterone have been described. An attractive nonchromatographic method is that of Brown et al. (64) for acid-hydrolyzed urine. The determination of urinary estriol by radioimmunoassay with a modification of a commercial kit for serum analyses was described by Kallner and Lantto (65). As many as 75 determinations/day can be performed by this method and the correlation with gas chromatography is high. Chattoraj et al. (66) assayed the less common urinary catechol estrogens by radioimmune methods. Two of the major metabolites of cortisol, tetrahydrocortisone and tetrahydrocortisol, as well as their 21-glucosiduronate conjugates were estimated by a simple radioimmunoassay by Will et al. (67). One of the main metabolites of corticosterone, tetrahydrocorticosterone, has also been estimated by radioimmunoassay (68). An important new assay in human pathology is the radioimmunoassay for urinary 17-hydroxyprogesterone, described by Wong et al. (69).

Protein-binding assays for the direct estimation of conjugated urinary steroids. Based on the work of Kellie et al. (70), who prepared an antiserum to testosterone 17β-glucosiduronate, Hennam et al. (71) have developed an assay for this compound in urine. A similar method has been described by Tresguerres et al. (72). Another group of steroids of interest has been estrogen glucosiduronates. Wright et al. (73) determined 3 specific estrogen glucosiduronates: estrone 3-glucosiduronate, estradiol 17β-glucosiduronate and estriol 16-glucosiduronate; and Baker et al. (74) determined not only these 3 but also estradiol 3-glucosiduronate and estriol 3-glucuronide. This was done directly, without pretreatment of the urine. Colins et al. (75) have assayed pregnanediol 3-glucosiduronate by radioimmune methods.

Enzyme immunoassays. Since the publications by Engvall and Perlmann (76), Avrameas and Guilbert (77) and Van Weemen and Schuurs (78) of early methods for protein-binding assays with enzymes instead of radioisotopes as labels in protein-binding assays, a rapid development has occurred in the field of enzyme-linked immunosorbent assay (ELISA). Subsequently, Rubenstein et al. (79) have described a "homogeneous" enzyme immuno-

assay which has developed into the commercially available EMIT system. A considerable number of enzyme immunoassay methods have been developed for steroids in blood, as reviewed by Schuurs and Van Weemen (80) and by Pratt (61).

Some of the more recently developed blood analyses for hormonal steroids, e.g., the progesterone assay by Joyce et al. (81), seem quite comparable in performance to radioimmunoassays. Since the instruments required are essentially a centrifuge and a colorimeter, this type of protein-binding assay of high specificity and sensitivity is accessible to the smaller laboratory. Allegedly, some of the enzyme immunoassay methods also have been used for urinary assays, but specific methodology for urinary hormonal steroids has not yet been worked out. The attractively simple homogenous enzyme immunoassay methodology has been applied to the estimation of cortisol in blood (82) and may be adapted also to urinary assays.

The enzyme immunoassay field, which is still in rapid development, holds great promise for the assay of individual urinary hormonal steroids in the near future. This type of assay has some clear advantages over radioimmunoassay. In particular, the enzyme label is usually stable over long periods of time, handling and disposing of radioisotopes is avoided, simple instrumentation can be used, and, because of the amplification effect of the enzyme label, greater sensitivities will perhaps ultimately be obtained.

Relative merits of commonly used assays for single steroid hormones in urine. In a world of high technology and computers, it is easy to forget that there are still sizable areas where laboratories may have little more than a colorimeter as their main instrumentation and, obviously, methods based on colorimetry will serve a useful function for some time to come. In more advanced laboratories, the choice between binding assays and other methods will depend a great deal on whether the laboratory performs only an occasional analysis, a short series of analyses as part of a scientific project, or whether a daily, more or less heavy, load of analyses can be expected. In the heavy load situation, binding assays of proven reliability would generally be preferable. For the occasional analysis, on the other hand, other methods would seem preferable because overall, they are more convenient, particularly if radioimmunoassays are used.

Where the highest specificity is required, without consideration for cost and effort, gas chromatography/mass spectrometry, which more and more serves as reference methodology in biochemical analyses, is clearly the method of choice.

The coming years should see a strong move toward the use of enzyme immunoassays. They will open the field of protein-binding immunoassays to the laboratory not possessing elaborate equipment and will also have great potential for the larger laboratory which is interested in establishing automated immunoassays for single hormonal steroids in urine.

PROFILE ANALYSES FOR STEROID HORMONES IN URINE

Active steroidal hormones usually are extensively transformed before their metabolites are excreted in the urine. The adrenocortical hormones, cortisol, e.g., is converted to more than 20 known metabolites; devising methods for their estimation thus becomes a complex undertaking. The analytical problem is further compounded because each metabolite may occur not only as the free steroid, but also conjugated in different ways, e.g., as glucosiduronates, sulfates, or combined conjugates. This makes the complete characterization of the urinary spectrum of steroid metabolites of hormonal steroids a formidable undertaking.

Nonautomated profile analyses for urinary steroid hormones. The ideal system for the analysis of profiles of hormonal steroids would be one that would allow the estimation of both the small amounts of free steroids present in the urine and, without hydrolysis, would also determine the different conjugates that form the bulk of the steroid metabolites excreted. Neither the early pioneering work of Schneider and his colleagues with paper chromatography (83,84) nor later work by A.E. Kellie's group (85) with column chromatography succeeded in arriving at practical methods for urinary steroid conjugates, although some separation of different conjugates in urinary extracts was achieved.

The latest approach by Kellie's group, an attempt at the direct assay of glucuronides by radioimmunoassay (74), is one possible means of analyzing at least the main conjugates of major urinary steroids. Otherwise, the steroid conjugates must be either hydrolyzed as completely as possible and then determined, or alternatively, the free steroids and conjugates of various kinds must be separated then each group hydrolyzed separately, and finally, the steroid content for each group determined.

The bulk hydrolysis has been used for routine analyses, and the more informative but much more complex group hydrolysis for research purposes. Once hydrolysis has been

achieved—usually by acid hydrolysis, solvolysis, and/or enzymatic cleavage—a separation into individual steroids ordinarily is performed. For this, all available types of chromatography have been used, as reviewed earlier by Bush (11), Neher (86) and, more recently, by Heftmann (12). The great number of chromatographic methods does not permit more than a few representative examples from each area. Of the many paper chromatographic systems for urinary steroids, the methods developed by Bush (11) have been widely used. Touchstone (87) has developed efficient systems for the estimation of, in particular, urinary estrogens by thin layer chromatography. For this type of analysis, high performance thin layer chromatography as described in a monograph by Zlatkis and Kaiser (88) holds considerable promise, although practical methods for urinary steroids have yet to be developed. Among the gas chromatographic methods, of which there are a great many, newer methods in which high-resolution capillary columns are used (89,90) are particularly impressive. The considerable power of combined gas chromatography/mass spectrometry as an analytical tool further enhanced by an initial separation into conjugate groups has been efficiently exploited by Swedish and Finnish workers (91,92) in their studies of urinary hormones, and this type of steroid analysis and its potential has been reviewed by Brooks and Middleditch (93).

One of the earliest and most successful applications of liquid column chromatography to the assay of urinary steroids was the Brown method (94) for the 3 main urinary estrogens. High-resolution liquid chromatographic systems for neutral hormonal steroids in urine have been reported by Seki et al. (95,96).

Semi-automated profile analyses for urinary steroid hormones. The widespread tendency toward the application of semi-automated and automated methodology in biochemical analyses and the introduction of data-handling equipment, increasingly coupled with dedicated minicomputers and miniprocessors, has also affected the field of urinary steroid analyses. Because of the great number of free steroids and conjugates present in urine, fully automated methods would either have to have very high resolution, if a direct separation of free steroids and conjugates were to be attempted, or the separations would have to be performed by, e.g., sequential automated chromatography. Alternatively, methods for the initial hydrolysis and preliminary extraction would have to be automated and final separation, e.g., by chromatography, and quantitation by endpoint analysis would have to be coupled with data-handling equipment. This last possibility could undoubtedly be realized with current knowledge and technology, but the need for this type of analysis has not been urgent enough for investment in the necessary apparatus. What has been developed at this time are various systems that greatly expedite profile analysis of urinary steroids by automating and, in some cases, computerizing important sections of these analyses.

Three systems for the semi-automated assay of urinary hormonal steroids have been developed as special projects with years of efforts behind each endeavor. They are the Steroid Analyzer system developed in Johnson's laboratory (97,98), the CASSANDRA system established by Bush (99-101) and the multicolumn system built in our laboratory (23).

The Steroid Analyzer system. The development of this system has been described by Johnson (102). The advantage of this system is the sophisticated computer control of gradient elution from silicic acid columns with varying contents of water. Computerized programs also have been developed for the selection of thin layer systems for separation of steroids in unresolved peaks. The system seems particularly useful for the in-depth study of steroids in biological mixtures. It is a low-capacity system.

The CASSANDRA system. In contrast, the stated purpose behind the paper chromatographic CASSANDRA (Chromatographic Automatic Soaking, Scanning and Digital Recording Apparatus) system developed by Bush (99) is to increase the capacity for chromatographic work dramatically, permitting the large-scale determinations of metabolite patterns in biological fluids. It is predicted that the emphasis in, e.g., clinical chemistry will switch from single substance analyses toward profile analyses, and automated equipment will be necessary for this task.

Bush has constructed the CASSANDRA apparatus as a solution to this problem. It has been used mostly for the analysis of urinary hormonal steroids, and is a most ingenious piece of instrumentation (99-101), consisting of a machine for treating chromatograms with liquid reagents and a scanner for paper strip chromatograms connected to a minicomputer for data handling. The system is capable of processing 500 paper chromatograms in a working day.

The multicolumn system. This system is similar in scope and purpose to the Bush system, but takes a somewhat different approach. It uses column chromatography for the separation because the higher resolution thus obtainable was considered critically important

for the separation of complex mixtures of substances, as exemplified by the steroids present in urine. To overcome the longer running times required before the microparticulate columns became available, simultaneous chromatography on a number of columns operated in parallel was introduced (103,104). This number was gradually increased from 6 to 25 (105), and the glass columns were changed to capillary Teflon columns (106), which were batch-fillable (107) and inexpensively replacable. Many components have undergone steady improvement over the years. In the current system (23), fractions from the capillary columns eluted by gradient elution chromatography are collected simultaneously in special multicollectors (108) and quantitation is performed in special multisectioned multicuvettes (109) using a spectrophotometer-computer interfaced system (110). We subsequently replaced the minicomputer used for data-handling and quantitation by a much less expensive microcomputer system that can be reasonably serviced by laboratory personnel.

This system has a high capacity, and thus we are able to handle several hundred liquid chromatograms in a working day. In principle, by adapting the system to high performance liquid chromatography, ultra-high capacity systems capable of handling 1,000 liquid chromatograms per working day would be conceivable. We have used the system for a detailed study of neutral steroid hormones in the urine (111).

Other Possible Approaches to Semi-automated and Automated Profile Analysis of Urinary Steroid Hormones

Sequential gas chromatography. Undoubtedly, such systems can be set up to perform automated chromatography. However, they have a fairly low capacity, since even a high-resolution capillary column gas chromatogram of urinary steroids takes more than 30 min to perform (89).

High performance liquid chromatography. In principle, such systems can also be established with automatic sampling and data handling systems, especially as microsystems using the principle of the FAMILIC-100 micro high performance liquid chromatograph (112) and, ideally, adapted to our multicolumn systems, these could become formidable systems for automated analyses of hormonal urinary steroids.

Combinations of chromatography and automated systems for immunoassay. An interesting system has been described by Sippell et al. (113) for mechanized Sephadex LH-20 multiple column chromatography as a preliminary step to automated multisteroid radioimmunoassays. It has been developed for blood assays, but similar systems should be possible for urinary steroid hormones. Obvious extensions of this type of system would be combined chromatographic and enzyme immunoassay systems, in which the colorimetric or fluorometric endpoint would make quantitation much easier than in radioimmunoassay methodology.

Mechanized continuous-flow multicolumn system. Methodology for some hormones in blood, based on a combination of simultaneous multicolumn chromatography on 25 columns and peristaltic pumps, has been introduced by Horn et al. (114). It has been developed for blood analyses and uses competitive protein binding assays for quantitation but, in principle, it is adaptable to the estimation of urinary steroid hormones, although profile analysis would perhaps be an inappropriate application for this system.

Conclusion

The field of profile analysis for urinary steroid hormones presents a fertile field for ingenuity. Either the development of high-resolution systems for the direct estimation of conjugates, or automated systems for the initial hydrolysis of conjugated hormonal steroids and extraction of the steroids released is needed at this time. Once the free steroids are on hand, many possibilities exist for the fast and accurate estimation of steroid metabolites in urine.

FUTURE OF THE ANALYSIS OF URINARY STEROID HORMONES

It is safe to predict that in the area of single-steroid and group assays the tested and proven simple colorimetric and fluorometric assays will continue to be used for years to come, but probably with a continuing trend toward semi-automated and automated assays in laboratories having a sizable load of such analyses to perform. A most interesting development is the gradual emergence of the enzyme immunoassay methodology. This promises to add to the colorimetric and fluorometric endpoint analyses the specificity of the immune reactions.

The convenient radioimmunoassay methodology, so suitable to large-scale batch analyses, will undoubtedly continue to flourish, particularly as more and more sophisticated, automated equipment with electronic data handling becomes available. But increasing competition can be expected from high-resolution gas chromatography and liquid chromatography.

In the field of high-specificity analyses, methods in which the mass spectrometer is used as a detector can be expected to dominate. This is now possible both in gas chromatography and high performance liquid chromatography. The development of routine instrumentation for such assays is feasible, and would, with fast chromatography, give very respectable capacities per technician and work day, if properly interfaced with computers.

For profile analyses, the greatest promise at this time is probably high performance liquid chromatography coupled with multicolumn methodology, but many other approaches may prove profitable.

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Techniques for the Isolation and Identification of Steroids in Insects and Algae*

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ABSTRACT

Analytical techniques, methods and instrumentation employed for the extraction, isolation, separation, purification and identification of steroids from algae and insects are presented. The techniques include adsorption and argentation column chromatography and counter-current distribution for separating the individual steroids, and thin layer chromatography and gas liquid chromatography for monitoring the purification process. Double bond, steric and alkyl substituent separation factors and relative retention times are reported for a large number of sterols on 4 different column systems. Their use permits the tentative structural assignment of sterols. Ultraviolet, infrared, nuclear magnetic resonance (NMR) and mass spectral analyses are discussed in light of their significance in the isolation and identification of steroids from insects and algae. Numerous examples are presented, including the use of 220 MHz NMR spectrometry, which permits the differentiation and characterization of C-24 epimeric sterols and allows for a semiquantitative estimate of the 24α- and 24β-epimers present in a mixture.

INTRODUCTION

Analytical techniques and instruments are now readily available for the separation, purification and identification of steroids in complex extracts from plants and animals, including insects. Thus, it has been possible to determine the sterol content of insects and algae and to study the metabolism of plant sterols and of ecdysteroids in insects, the processes involved in algal sterol biosynthesis and the inhibition of sterol metabolism and biosynthesis in both insects and algae.

Our laboratories have employed specific procedures for the extraction, isolation and separation of sterols from insects and algae. Once the sterols are isolated, various methods such as adsorption and argentation column chromatography and counter-current distribution (CCD) are employed to separate the individual sterols, and thin layer chromatography (TLC) and gas liquid chromatography (GLC) are available for monitoring the process of purification. For positive identification of the individual sterols, we have employed a combination of analytical tools such as GLC on 3 or 4 different stationary phases for tentative structural assignment. Ultraviolet (UV) and infrared (IR) analyses have been used to determine the various chromophores and functional groups in steroids and also for making direct comparisons. Mass spectrometry (MS) in conjunction with gas liquid chromatography (GC-MS) is a vital tool in the examination of sterol extracts and in the characterization of steroids, especially at the microgram level. Proton magnetic resonance (PMR) spectroscopy, because of the equivalence of the 3 protons of certain methyl groups of steroids that appear as sharp peaks in the PMR spectra, has served us well in determining the stereochemistry and configuration of sterols at C-24 and the environment around the methyl groups of ecdysteroids. For example, the differences in chemical shifts of methyl signals of ecdysteroids have been used to advantage in the elucidation of their structures. The aforementioned instrumental methods and techniques will be discussed in light of their significance in the isolation and identification of steroids from insects and algae. Examples of extraction and isolation procedures will be presented.

PROCEDURES

Instrumentation

A Virtis homogenizer and a Waring Blender (3.8-liter capacity) were used to homogenize small and large quantities of insect tissues, respectively. An International Model PR-2 refrigerated centrifuge was used to centrifuge extracts. Ultraviolet spectra were taken in hexane or methanol with a Bausch and Lomb Spectronic 505 spectrophotometer. Nuclear (NMR) magnetic resonance (NMR) spectra were recorded at 60 and/or 100 and 220 MHz with a Varian A-60A, HA-100 or HR-200 NMR

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spectrometer, respectively, using deuterated chloroform or deuterated pyridine as solvent and tetramethylsilane (TMS) as internal NMR standard. Mass spectra were recorded on an LKB Model 9000 GC-MS (LKB-Produkter AB, Stockholm). The compounds were introduced into the ion chamber either directly or from the effluent of 0.75% SE-30 GLC column. The ionization energy was 70 eV. GLC analyses were made on a Barber Colman Model 10 Chromatograph and a Glowall Chromalab Model A-110 Chromatograph. Both instruments were equipped with an Argon-ionization detector and argon was used as carrier gas. Column packings were prepared from 100-120-mesh Gas Chrom Q and P, according to Horning et al. (1). The liquid phases used were 1% QF-1, 3% Hi-EFF-8BP, 0.75% and 3% SE-30, and 2% PMPE.

Analab Anasil H was used for routine TLC analysis; for thin layer argentation chromatography, we prepared Silica Gel H plates of 250-μ thickness from a slurry of 25 g of Silica Gel H in 65 ml of aqueous silver nitrate (12.5% w/v) according to a previously reported method (2). More recently we employed a newly developed technique (3) that drastically reduced the quantity of silver nitrate needed: Analab Anasil H plates were dipped into a 4% silver nitrate solution prepared by a published method (3). IR spectra were obtained with a Perkin-Elmer Model 221 prism-grating spectrophotometer.

ALGAL STEROLS

Sterol Extraction and Isolation from Algae

The algal cells were harvested in a Sharples Super Centrifuge and freeze-dried. The dried cells were ground to pass a 40-mesh screen and extracted with chloroform/methanol (2:1) in a Soxhlet apparatus for 24 hr. The solvent was removed under vacuum and the lipid material was saponified with a 20% solution of potassium hydroxide in 80% aqueous ethanol. The nonsaponifiable material was then extracted with ether for 12 hr in a liquid-liquid extraction apparatus (4), followed by the evaporation of the ether under nitrogen. The nonsaponifiable lipid was fractionated as described (5) on Woelm Neutral Alumina (Woelm, ICN Pharmaceuticals, Cleveland, OH), Activity Grade II. The sterols were eluted in the ether fraction.

In certain cases, sterols were precipitated from the nonsaponifiable fraction with digitonin, the digitonide was split with dimethyl sulfoxide and the sterols were extracted into *n*-hexane (6).

Separation of Dimethyl, Methyl and Desmethyl Sterols

To separate dimethyl, methyl and desmethyl sterols, the sterols were chromatographed on a column of neutral alumina, Activity Grade II, and eluted with increasing concentrations of ether in *n*-hexane (7). Fractions containing 0, 10, 20, 30, 40, 50, 70 and 90% ether in *n*-hexane and 100% ether were collected. The 20% ether, 30% ether and 90% ether in hexane yielded the dimethyl, methyl and desmethyl sterols, respectively (7). Fractions were analyzed by TLC and GLC.

Separation of Saturated and Unsaturated Steryl Acetates

After acetylation (pyridine-acetic anhydride [3:1] overnight at 23 C), the steryl acetates were further separated according to the degree of unsaturation by chromatography (2) on a column of 20% silver nitrate-impregnated Unisil (100-200-mesh, Clarkson Chemical Co., Williamsport, PA). Fractions containing 0, 10, 20 and 50% benzene in hexane and 100% benzene were collected. More recently, we have chromatographed the acetates on 20% silver nitrate-Unisil by eluting the column with increasing concentrations of 1, 2, 3, 4, 5, 6, 7 and 9% ether in hexane (8). The fractions were monitored by GLC and TLC (silver nitrate-silica gel). Purified sterols or their acetates were further analyzed by UV, IR, PMR and MS, including GC-MS analyses.

INSECT STEROLS AND ECDYSTEROIDS

Extraction and Isolation of Sterols from Insects

Material was amassed by freezing insects at the egg, larval, prepupal, pupal or adult stages. The lipids were extracted with chloroform/methanol (2:1) using a Virtis homogenizer and then fractionated on Unisil (20 ml lipid/g adsorbent) using a modification of the Goodman and Shiratori elution system (9). With a 25-g column (2 x 14 cm), a typical elution scheme was as follows: 150 ml 10%, 175 ml 21%, 375 ml 72% benzene in *n*-hexane, 75 ml benzene, 375 ml chloroform and 250 ml methanol. The fractions were monitored by TLC. In samples containing unstable sterols, such as those containing the 5,7-diene system, the extracts and column fractions were stored under refrigeration in an atmosphere of nitrogen or in solutions of benzene-methanol, and fractions were protected from light during chromatography.

The 21% benzene-in-hexane fraction con-

tained the steryl esters and the chloroform fraction contained the free sterols. The steryl esters were saponified in 4% methanolic potassium hydroxide for 2-4 hr at reflux temperature or at 65 C. The nonsaponifiable was chromatographed as before, or on neutral alumina, Activity Grade II (5). The free sterols and sterols from steryl esters were then sufficiently pure for GLC analyses.

For stable sterols, neutral alumina, activity Grade II, could be used in place of the Unisil column. In such cases, the lipids were chromatographed in a ratio of 1 g/30 g of alumina on a 2-cm id column, eluted typically with 200-ml fractions of each: hexane, hexane/benzene (1:1), benzene and ether. The hexane/benzene (1:1) fraction contained the steryl esters and the ether fraction contained the free sterol.

Separation of Saturated and Unsaturated Sterol Acetates

The insect sterols were acetylated and separated according to the degree of unsaturation on a 20% silver nitrate-Unisil column.

Extraction, Isolation and Identification of Ecdysteroids from an Insect

The extraction, isolation and identification of ecdysteroids from insects are generally very lengthy and tedious processes. A detailed procedure for the ecdysteroids in the tobacco hornworm (*Manduca sexta* L.) during pupal-adult development, 5 days after the peak titer of molting hormone activity can be found elsewhere (10). In this paper, we have condensed the procedure to a flow diagram (Scheme I) and a table (Table I). The 1.5 g of material containing 93% of the total molting-hormone activity was chromatographed on silicic acid and the results are presented in Table I. The ecdysteroids eluted in fractions 3 and 4 were further separated and purified by CCD, TLC and microcolumn chromatography, and identified as previously reported (10).

DISCUSSION

In certain instances, our methods may appear to be time-consuming and unnecessary and there may be instances where one could alter or improve certain steps without loss in material or efficiency. For example, we have not had any difficulty in conducting GLC or mass spectral analyses on the free sterols. The only sterol derivative we have used for our sterol analyses is the acetate, which is very stable and can be quantitatively prepared and determined, like the free sterol. We routinely subject all newly isolated sterols, as their acetates, to both argentation TLC and column chromatography for the separation of the saturated and unsaturated sterols having one, 2 or 3 double bonds. A large number of sterols differing in degree of unsaturation or E and Z pairs cannot be readily distinguished from each other or separated well enough by GLC on an SE-30 column. For example, cholestanol and cholesterol, campesterol and 24-methylene-cholesterol, Z-22-dehydrocholesterol and E-22-dehydrocholesterol, desmosterol and 7-dehydrocholesterol, coprostanol and 5β-cholest-7-en-3β-ol, and pollinastanol and 14α-methyl-5α-cholest-7-en-3β-ol are pairs not readily separated by GLC on an SE-30 column, or for most practical purposes, on any of 4 different GLC systems (Table II). However, most of them are separable by argentation column chromatography and TLC.

Because the various ecdysteroids have different partition coefficients, CCD has permitted the separation and almost total recovery of ecdysteroids of insects without destruction, and thus has served well in the partial purification of small or large quantities of these materials. A number of CCD solvent systems have been used for the separation of

Frozen hornworms (10.5 kg)
↓ Thawed overnight in methanol (0.5 ml/g of tissue), homogenized, centrifuged, filtered, residue re-homogenized twice with 75% methanol (0.25 ml/g of tissue), filtered.
Methanol and 75% methanol extracts
↓ Concentrated under vacuum, residue precipitates, ← precipitate rinse with 75% methanol.
Oil that separates partitioned beteen 70% meth- ← anol/hexane 70% methanol phase.
Methanol removed under vacuum.
Residue
↓ Partitioned between butanol/water, butanol phase rinsed with 2% sodium carbonate solution, centrifuged. Upper layer (butanol) retained and concentrated.
Active extract (butanol)
↓ Partitioned between 70% methanol/hexane, lower layer (70% methanol) retained and concentrated under vacuum.
Active extract
↓ Dissolved in methanol, stored at -10 C, centrifuged, precipitate rinsed with methanol, precipitate discarded, methanol retained and concentrated.
Active extract (3.7 g)
Dissolved in methanol, filtered through 50 g Woelm Neutral Alumina (Activity Grade I + 20% water).
↓ 800 ml of methanol collected, followed by 800 ml ← of 75% methanol.
Methanol concentrated under vacuum.
Active extract, 1.5 g

SCHEME I. Flow diagram of the isolation of ecdysteroids from tobacco hornworms during pupal-adult development (10).

the ecdysteroids besides those we have employed (11).

Where sufficient quantities of steroids were available, we have made use of all available instruments in our laboratories for the identification of new compounds so that a compendium of data is available to others for future positive identification. We can only give a few examples and demonstrate how GLC, UV, IR, NMR and MS analyses have served our laboratories in the identification of steroids from insects and algae. Each of these analytical tools will be discussed separately.

Gas Liquid Chromatography

Since the discovery in 1960 that steroids can be eluted from GLC columns with concentrations of liquid stationary phases of 1% or less at temperatures below 250 C, GLC has become an important tool for their separation and identification (12). The use of retention times relative to an internal standard (RRT), such as cholestane, has made it possible to correlate data obtained in different laboratories. Cholestane has been the internal standard most often used in analyses of sterols from insects because cholesterol is frequently the major sterol. However, in work on algae or plant sterols, cholesterol and cholesteryl acetate have been used as the internal standards for sterols and steryl acetates, respectively. Shortening the interval between the elution of the internal standard and the unknown makes the RRT more reproducible, especially in instruments with slight temperature fluctuations. In our laboratories, sterols are analyzed as free sterols and steryl acetates, because both forms give satisfactory results on all columns. Another practical reason for this is that sterols are normally either isolated or obtained from the unsaponifiable material in the free state.

RRT relative to cholesteryl acetate have been obtained on 4 different columns for more than 90 sterols and related compounds (13). A number of these are listed in Table II. The results indicate that at least 3 columns are necessary for tentative identification of a sterol by GLC (13). Thus, we have always used at least 3 different columns for comparative studies and for structural determination.

With few exceptions, the RRT of a sterol vs cholesterol is identical to that of the steryl acetate vs cholesteryl acetate. The exceptions are important in relation to their structures. For example, for free sterols with a methyl group at C-4, the RRT is 0.04-0.09 (ca. 3-4%) higher than the corresponding acetate values. Free sterols with 2 methyls at C-4 have RRT values 0.10-0.15 (ca. 6-8%) higher than the corresponding acetates. These data have been very helpful in the GLC identification of biosynthetic intermediates, many of which contain one or 2 methyls at C-4. The only free sterols lacking methyl groups at C-4 that do not have the same RRT as the acetates are sterols of the coprostane (5β) series and 3α-hydroxy sterols. Each of these also has a higher RRT value as the free sterol than as the acetate.

TABLE I

Distribution of Mass and Biological Activity following Column Chromatography on Silicic Acid[a] of the Methanol Eluate from Alumina

Fraction	Volume (ml)	Mass (mg)	Total biological activity (HFU)[b]	Ecdysteroids eluted
Benzene/methanol (95:5)				
1	1200	438.6	-	
2	400	21.7	1.2×10^4	
Benzene/methanol (90:10)				
3	800	177.0	6.0×10^5	3-*epi*-Ecdysone Ecdysone 3-*epi*-20-Hydroxyecdysone 20-Hydroxyecdysone
Benezene/methanol (75:25)				
4	400	206.5	7.0×10^4	3-*epi*-20-Hydroxyecdysone 20-Hydroxyecdysone 3-*epi*-20,26-Dihydroxyecdysone 20,26-Dihydroxyecdysone
Methanol				
5	400	534.2	1.0×10^4	

[a]Column 4.4 cm (id) X 2.5 cm, 18 g of silicic acid.

[b]House fly unit is equivalent to that quantity of pure hormone that causes 50-60% puparium formation in house fly larvae (for ecdysone 0.003 μg = 1 HFU).

TABLE II

Relative Retention Times of Steryl Acetates[a]

Steryl acetate	Gas chromatographic system			
	SE-30[b]	QF-1[c]	Hi-Eff 8BP[d]	PMPE[e]
5β-Cholest-7-en-3β-ol	0.86 (0.92)	0.88	0.76	0.77
5β-Cholestanol	0.86 (0.93)	0.90	0.76	0.74
Z-22-Dehydrocholesterol	0.87	0.88	0.88	0.87
5α-Cholestan-3α-ol	0.91 (0.99)	0.93	0.80	0.82
E-22-Dehydrocholesterol	0.91	0.89	0.91	0.92
5,22,24-Cholesta-trien-3β-ol	0.94	0.91	1.35	1.37
5,7,22-Cholesta-trien-3β-ol	0.99	1.00	1.21	1.14
Cholesterol	1.00	1.00	1.00	1.00
5α-Cholestanol	1.03	1.05	1.00	1.02
14α-Methyl-5α-cholest-8(14)-en-3β-ol	1.04	1.08	0.94	0.94
5,25-Cholesta-dien-3β-ol	1.07	1.12	1.31	1.27
Desmosterol	1.09	1.12	1.29	1.29
5,7-Cholesta-dien-3β-ol	1.09	1.12	1.29	1.09
Brassicasterol	1.12	1.09	1.10	1.09
5α-Cholest-7-en-3β-ol	1.12	1.11	1.21	1.26
Zymosterol	1.13	1.09	1.30	1.39
Pollinastanol	1.16	1.23	1.16	1.17
14α-Methyl-5α-cholest-7-en-3β-ol	1.17	1.21	1.17	1.16
Ergosterol	1.22	1.22	1.44	1.33
24-Methylenecholesterol	1.26	1.28	1.43	1.39
Lophenol	1.27 (1.32)	1.25	1.32	1.32
14α-Methyl-5α-cholesta-7,22-dien-3β-ol	1.29	1.30	1.26	1.25
Campesterol	1.30	1.29	1.32	1.29
Campestanol	1.34	1.35	1.30	1.32
5,7-Ergosta-dien-3β-ol	1.42	1.45	1.69	1.60
Stigmasterol	1.42	1.32	1.34	1.32
24-Methylenepollinastanol	1.46	1.58	1.68	1.63
7-Ergost-en-3β-ol	1.46	1.42	1.59	1.63
24-Methylpollinastanol	1.51	1.61	1.53	1.52
14α-Methyl-5α-ergost-8-en-3β-ol	1.52	1.53	1.52	1.49
Lanosterol	1.54 (1.66)	1.62	1.47	1.41
Spinasterol	1.58	1.46	1.61	1.65
Cycloartanol	1.61 (1.71)	1.73	1.45	1.40
Sitosterol	1.63	1.56	1.60	1.54
Fucosterol	1.63	1.50	1.76	1.68
Stigmasterol	1.67	1.62	1.58	1.57
28-Isofucosterol	1.69	1.55	1.85	1.79
Cycloartenol	1.75 (1.87)	1.85	1.88	1.81
5α-Stigmast-7-en-3β-ol	1.83	1.71	1.93	1.93
24-Methylcycloartanol	2.06 (2.21)	2.24	1.89	1.79

[a]Relative to cholesteryl acetate, values in parentheses are for free sterols relative to free cholesterol. When no value is given, it is the same as the acetate value.

[b]3% SE-30 on Gas Chrom Q, 244 C, 20 psi, argon flow rate 150 ml/min.

[c]1% QF-1 on Gas Chrom P, 231 C, 20 psi, argon flow rate 50 ml/min.

[d]3% Hi-Eff 8BP on Gas Chrom Q, 238 C, 25 psi, argon flow rate 95 ml/min.

[e]2% PMPE on Gas Chrom Q, 250 C, 20 psi, argon flow rate 95 ml/min.

A partial list (for a more complete list, see Ref. 13) of double bond and steric separation factors of sterols and the separation factors for various alkyl substituents of the sterol molecule may be found in Tables III and IV, respectively. None of the 4 GLC systems gives complete separation of the Δ^5-sterols from the corresponding stanols, but they can be easily distinguished from each other, except on Hi-Eff 8BP. The agreement in RRT determined in duplicate experiments, is usually within 1%. All 4 GLC systems separated Δ^7-sterols from stanols and $\Delta^{5,7}$-sterols from stanol-5, but the separation is more complete on Hi-EFF 8BP and PMPE. The effect of the Δ^{22}-unsaturation in reducing the retention time of a sterol is lessened in a sterol with an 8-carbon side chain compared to that of a sterol with a 9- or 10-carbon side chain. The effect of unsaturation at C-22 is consistent for a large number of compounds tested, regardless of the type of column. Only compounds containing a double bond at C-24 or C-25 in addition to that at C-22 show a change in the separation factor for the Δ^{22} unsatu-

ration.

The Δ^{25}-sterol is eluted faster than the corresponding saturated sterol on SE-30 and QF-1, but it is eluted more slowly on Hi-Eff 8BP and PMPE. As with Δ^{22}-sterols, the retention characteristics of the Δ^{25}-sterol are affected by an alkyl group at C-24 and additional unsaturation in the side chain. Sterols with a double bond at C-24 are separated on QF-1 and SE-30, but are separated more completely on Hi-Eff 8BP and PMPE. Again, additional unsaturation in the side chain (e.g., at C-22) changes the retention characteristics of the Δ^{24}-sterol.

The presence of the $\Delta^{24(28)}$-double bond has little effect on the retention time of a sterol on QF-1 or SE-30, but it significantly increases the retention time on Hi-Eff 8BP and PMPE. Only slight differences are noted between sterols with 9- or 10-carbon side chains. When the *trans*-$\Delta^{24(28)}$ configuration is present, as in 28-isofucosterol, the retention time in all GLC systems is slightly greater than when the *cis*-$\Delta^{24(28)}$ configuration is present, as in fucosterol.

The effect of alkyl substituents in the side chain on GLC retention times is quite similar for both polar and nonpolar stationary phases. Regardless of which GLC system is used, a C-24 methyl substituent increases the retention time from 1.28 to 1.31 times (Table IV). Interaction between the unsaturation at C-22 and the alkyl substituent at C-24 results in a difference in separation factors between C-24 methyl sterols with and without a C-22 double bond. For example, on SE-30, the RRT of brassicasterol (ergosta-5,22-dien-3β-ol) relative to that of E-22-dehydrocholesterol is 1.23 whereas the RRT of campesterol relative to that of cholesterol is 1.30. The same effect is seen in the separation factors for the C-24 ethyl sterols.

The presence of a 4α-methyl group does not increase the retention time as much as a C-24 methyl, possibly because of interaction between the 4α-methyl and the 3β-hydroxyl or 3β-acetoxy groups. This interaction is the basis for the GLC method (13) of distinguishing between 4,4-dimethyl, 4-methyl and 4-des-

TABLE III

Double Bond and Steric Separation Factors of Sterols

Substituent	Sterol compared	Carbons in sterol	Carbons in side chain	Gas Chromatographic Systems			
				SE-30	QF-1	Hi-Eff 8BP	PMPE
Δ^5	Δ^5/stanol (5α)	27	8	0.97	0.95	1.00	0.98
		28	9	0.97	0.96	1.01	0.98
		29	10	0.97	0.96	1.01	0.98
Δ^7	Δ^7/stanol (5β)	27	8	1.00	0.98	1.00	1.04
	Δ^7/stanol (5α)	27	8	1.09	1.06	1.21	1.23
		28	9	1.09	1.05	1.22	1.23
		29	10	1.09	1.06	1.22	1.23
$\Delta^{5,7}$	$\Delta^{5,7}$/stanol (5α)	27	8	1.06	1.07	1.29	1.20
		28	9	1.06	1.07	1.30	1.21
		29	10	1.06	1.08	1.30	1.20
Δ^{22} (Z)	$\Delta^{5,22}/\Delta^5$	27	8	0.87	0.88	0.88	0.87
Δ^{22} (E)	$\Delta^{5,22}/\Delta^5$	27	8	0.91	0.89	0.88	0.87
	$\Delta^{5,7,22}/\Delta^{5,7}$	27	8	0.91	0.89	0.94	0.93
	$\Delta^{5,22,24}/\Delta^{5,24}$	27	8	0.86	0.83	1.05	1.06
	$\Delta^{5,22}/\Delta^5$	28	9	0.86	0.84	0.83	0.84
	$\Delta^{5,7,22}/\Delta^{5,7}$	28	9	0.86	0.84	0.85	0.83
	$\Delta^{7,22}/\Delta^7$ 5α	28	9	0.86	0.84	0.83	0.84
	$\Delta^{7,22}/\Delta^7$ 5α	29	10	0.85	0.85	0.83	0.84
	$\Delta^{5,22}/\Delta^5$	29	10	0.87	0.85	0.84	0.86
	$\Delta^{5,7,22}/\Delta^{5,7}$	29	10	0.87	0.84	0.84	0.86
$\Delta^{8(9)}$	$\Delta^{8(9)}$/stanol (5α)	27	8	1.02	0.96	1.01	1.05
		28	9	1.02	0.95	1.02	1.05
$\Delta^{8(14)}$	$\Delta^{8(14)}$/stanol (5α)	29	10	0.96	0.93	0.96	0.97
Δ^{25}	$\Delta^{5,25}/\Delta^5$	27	8	1.07	1.12	1.31	1.27
	$\Delta^{5,25}/\Delta^5$	29	10	0.95	0.94	1.05	1.03
Δ^{24}	$\Delta^{5,24}/\Delta^5$	27	8	1.09	1.09	1.29	1.29
	$\Delta^{5,22,24}/\Delta^{5,22}$	27	8	1.03	1.01	1.48	1.49
$\Delta^{24(28)}$	$\Delta^{5,24(28)}/\Delta^5$	28	9	0.97	0.99	1.08	1.08
	$\Delta^{7,24(28)}/\Delta^7$ (5α)	28	9	0.97	0.99	1.09	1.08
	$\Delta^{24(28)}$/stanol (5α)	29	9	0.97	0.98	1.10	1.07
	$\Delta^{5,24(28)}/\Delta^5$	29	10	1.00	0.96	1.10	1.09
$\Delta^{5,24(28)}$ (iso)	$\Delta^{5,24(28)}/\Delta^5$	29	10	1.04	0.99	1.16	1.16
	$\Delta^{7,24(28)}/\Delta^5$	29	10	1.03	0.99	1.15	1.16

TABLE IV

Alkyl Substituent Separation Factors of Sterols

Substituent	Double bonds	Carbons in sterols	Gas Chromatographic Systems			
			SE-30	QF-1	Hi-Eff 8BP	PMPE
24-Methyl	$\Delta^{5,22}$	28/27	1.23	1.22	1.21	1.18
	$\Delta^{5,7,22}$		1.23	1.22	1.19	1.17
	Δ^{5}		1.30	1.29	1.32	1.26
	Δ^{7}		1.30	1.28	1.31	1.29
	$\Delta^{5,7}$		1.30	1.29	1.31	1.30
	Δ^{7}	29/28	1.30	1.27	1.30	1.28
	stanol	28/27	1.30	1.29	1.30	1.29
24-Ethyl	$\Delta^{5,22}$	29/27	1.54	1.48	1.47	1.43
	Δ^{5}		1.63	1.56	1.60	1.54
	Δ^{7}		1.63	1.54	1.60	1.53
	$\Delta^{5,7}$		1.63	1.56	1.54	1.53
	$\Delta^{8(9)}$		1.63	1.54	1.58	1.54
24-Ethyl/24-methyl	$\Delta^{5,22}$	29/28	1.27	1.21	1.22	1.21
	$\Delta^{7,22}$		1.26	1.22	1.22	1.21
	$\Delta^{5,7,22}$		1.26	1.20	1.20	1.21
	Δ^{5}		1.25	1.21	1.21	1.19
	Δ^{7}		1.25	1.20	1.21	1.18
	$\Delta^{5,7}$		1.25	1.21	1.21	1.18
	stanol		1.25	1.20	1.22	1.19
4-Methyl	stanol	29/28	1.16	1.14	1.09	1.04
	$\Delta^{7,24(28)}$		1.13	1.12	1.09	1.04
	$\Delta^{8(9)}$		1.14	1.13	1.08	1.02
	Δ^{7}	28/27	1.14	1.11	1.09	1.05
14-Methyl	$\Delta^{8(9)}$	29/28	0.99	1.07	0.93	0.88
	Δ^{7}	28/27	1.04	1.09	0.97	0.92
	$\Delta^{7(22)}$	29/28	1.03	1.08	0.95	0.91
4,4' Dimethyl/ methyl	stanol	30/29	1.19	1.24	1.14	1.15

methyl sterols. Partial shielding by the polar 3β-hydroxyl or 3β-acetoxy may also explain why the 4-methyl adds little to the RRT on the Hi-Eff 8BP and PMPE columns. The presence of a second methyl at C-4 increases the retention time of a sterol more on a nonpolar than on a polar liquid phase (19 and 24% for SE-30 and QF-1, respectively, vs 14 and 15% for Hi-Eff 8BP and PMPE, respectively).

The effect of a 14α-methyl group on RRT is unexpected. On 3 columns, the presence of a 14α-methyl group in a $\Delta^{8(9)}$-sterol actually *decreases* the retention time. It is apparent that the location of the nuclear double bond can alter the contribution of the 14α-methyl group to the RRT of the sterol. In Δ^{7}-sterols, the 14α-methyl group increases the retention time on SE-30 as well as on QF-1.

Additional separation factors can be calculated for other structural features, using the data given in Table II. Using the separation factors in Tables III and IV, we can calculate the probable RRT of scores of sterols which may not have been isolated or synthesized to date. Over 12 previously unidentified sterols, thought to be intermediates in sterol biosynthesis in *Chlorella*, have been tentatively identified in this way (14), even before the usual methods of identification had been used.

Ultraviolet Spectra

Ultraviolet (UV) spectroscopy has long been recognized as a useful tool for the detection and identification of trace amounts of compounds having chromophoric systems. Empirical correlations of UV absorption with structure have afforded a powerful means of structure elucidation of steroids (15,16). UV spectroscopy was used for the early detection of the metabolic conversion of cholesterol to 7-dehydrocholesterol and the isolation of the 7-dehydrocholesterol from the German cockroach (*Blattella germanica* L.) as well as other insects and certain specific tissues of insects (17). The homoannular diene of the B-ring of 7-dehydrocholesterol with λ_{max} at 272, 282 and 294 nm and ϵ of 10,450, 11,000 and 6,200, respectively, readily identifies this system. This absorption spectrum also aided in the isolation and identification of ergosta-5,7-diene-3β-ol and 24R-stigmasta-5,7,22-trien-3β-ol from triparanol-inhibited algae (18).

The heteroannular dienes have extinction

coefficients that are usually higher and their λ_{max} are in the 220-250 nm range. From *Cholorella sorokiniana*, cultured in the presence of triparanol succinate, we have isolated and identified 5α-ergosta-8,14-dien-3β-ol, λ_{max} 251, ε 18,000. In elucidating the pathway for the conversion of stigmasterol to cholesterol in the tobacco hornworm, UV analyses readily indicated the presence of a conjugated diene system that exhibited a strong peak at 240 nm with shoulders at 234 and 247 nm. This led to the identification of E-22-cholesta-5,22,24-trien-3β-ol (19) as an intermediate in the conversion of stigmasterol to cholesterol in this insect.

The UV spectra of the molting hormones of insects and ecdysteroids of plants are characterized by an intense UV absorption bond with a λ_{max} at ca. 240-245 nm (in methanol) and ε 11,000-14,000 (11). This is typical of a compound with a 7-en-6-one chromophore. Thus, UV analyses have been used to follow the process of purification and for determining the purity of ecdysteroids. Ecdysteroids can be readily analyzed by UV for the presence or absence of the 14-hydroxyl group simply by heating the ecdysteroid with 0.5 N hydrochloric acid solution. In the presence of acid, there is an elimination of the 14-hydroxyl group, resulting in the formation of a conjugated dienone (λ_{max} 293 nm) and a nonconjugated ketone with a conjugated diene system, λ_{max} 244 nm (11).

Infrared Spectra

An infrared (IR) spectrum gives considerable information about a sterol and can be used to differentiate 2 sterols and to reveal the structure of a new sterol. Thus, IR analyses are very useful in characterizing the oxygen-containing functional groups and establishing the presence, location and type of unsaturation in a sterol and also in determining the stereochemical configuration at certain positions.

The IR spectra of cholestanol, cholesterol, desmosterol and lathosterol (5α-cholest-7-en-3β-ol) are shown in Figure 1. All 4 compounds can be distinguished from each other in their fingerprint region. The IR spectra of both cholestanol and lathosterol, which contain *trans*-A/B ring systems, differ from those of cholesterol and desmosterol in the 1000-1050 cm^{-1} region. In the region where the trisubstituted ethylene absorption band (800-850 cm^{-1}) appears, cholestanol shows only weak absorption whereas cholesterol, lathosterol and desmosterol, all of which contain trisubstituted double bonds, show sharp peaks in the 800-850 cm^{-1} region. Yet, the patterns of all 3 are different and can be differentiated from each

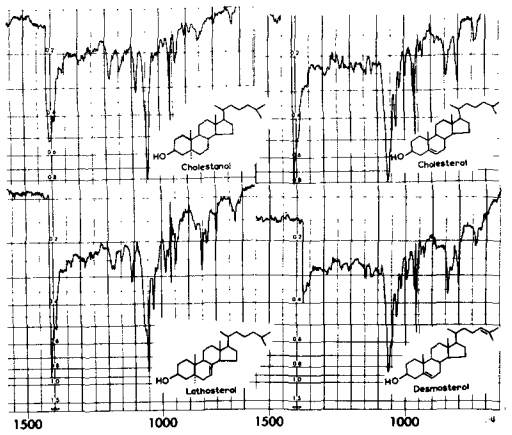

FIG. 1. Infrared spectra of cholestanol, lathosterol, cholesterol and desmosterol from 1500-600 cm^{-1}.

other in this region. The presence of another trisubstituted double bond at C-24 in desmosterol causes an increase in the absorption band at 828 cm^{-1} and it is this peak in the IR spectrum that readily differentiates desmosterol from cholesterol.

The IR spectra of C_{28}- and C_{29}- Δ^5-sterols with saturated side chains (campesterol, sitosterol and their C-24 isomers) do not distinguish these compounds from cholesterol or from one another. However, the combination of IR, PMR and GLC analyses leaves no doubt as to the identity of these sterols. The same is true for C_{28}- and C_{29}- Δ^7-sterols with saturated side chains.

The IR spectrum of brassicasterol, stigmasterol, poriferasterol or any sterol with a Δ^{22}-E disubstituted ethylene shows a very strong peak in the 965 cm^{-1} region, whereas the IR spectra of sterols with a $\Delta^{24(28)}$-monosubstituted ethylene (e.g., 24-methylenecholesterol, 24-methylenepollinastanol show a strong peak in the 885-895 cm^{-1} region. The IR spectra of 25-dehydrosterols with monosubstituted ethylenes also show IR absorption bands in this region. IR spectra, as first observed by Dusza (20), readily distinguish between the $\Delta^{24(28)}$-*trans* configuration of 28-isofucosteryl acetate and the $\Delta^{24(28)}$-*cis* configuration of fucosteryl acetate in the 800-850 cm^{-1} region. The IR spectra of the ecdysteroids show the characteristic α,β-unsaturated ketone absorption bands at 1640-1667 cm^{-1} and hydroxyl absorptions at 3300-3600 cm^{-1}.

Hence, IR spectroscopy is still of great importance for any laboratory concerned with the isolation, synthesis and structural identification of steroids and related natural products. We have only mentioned or discussed one

of the 2 types of noncarbonyl functional groups most commonly encountered in steroids, the ethylenic double bond, in order to illustrate the value of IR spectroscopy in steroid analyses.

Theoretically, the IR spectrum of each steroid is unique and, if the compound under study has been synthesized previously, its identity can be largely established by comparison with the spectrum of the authentic sample. Our laboratories have been involved in the isolation and identification of steroids over a period of years and have accumulated a large collection of steroid spectra which has enhanced the identification and structural elucidation of new steroids. However, in the initial period of our research, atlases of IR absorption spectra of steroids (21,22) were of considerable value, and others may find this is true, as well. Sadtler Research Laboratories also supplies a collection of IR spectra of steroids.

Mass Spectral Analyses of Sterols

Mass spectrometry, in conjunction with gas chromatography (GC-MS) has played a vital role in the analysis and elucidation of the structures of minute samples of sterols that are separable only by GLC. However, in most of our studies we have used MS as a diagnostic tool in conjunction with other instruments in the verification of molecular weights and tentative structures.

In elucidating the pathway in the conversion of ^{3}H-campesterol to cholesterol in the tobacco hornworm, we have isolated a radiolabeled steryl acetate with an IR spectrum showing strong bands at 1645 and 885 cm^{-1}, typical of a *gem*-disubstituted ethylene, which was identical with that of 24-methylenecholesteryl acetate. The GS-MS of this steryl acetate and 24-methylenecholesteryl acetate were identical. As is typical for Δ^5-steryl acetates, the M^+ peak was absent from both spectra, and the highest mass peak appeared at m/e 380 (M-60). Also, the second most abundant peak in both spectra occurred at m/e 296 (M-60-84), indicating a loss of acetic acid and the expected side-chain cleavage of the 22-23 bond, accompanied by a hydrogen transfer, thus identifying this compound as 24-methylenecholesteryl acetate (23). Similarly, fucosterol was identified as a metabolite in the conversion of sitosterol to cholesterol in the tobacco hornworm. The base peak in the mass spectrum of fucosterol also occurred at m/e 296 (M-18-98), indicating a loss of water and the expected side-chain cleavage of the 22-23 bond, accompanied by a hydrogen transfer (24).

In studies concerned with the conversion of

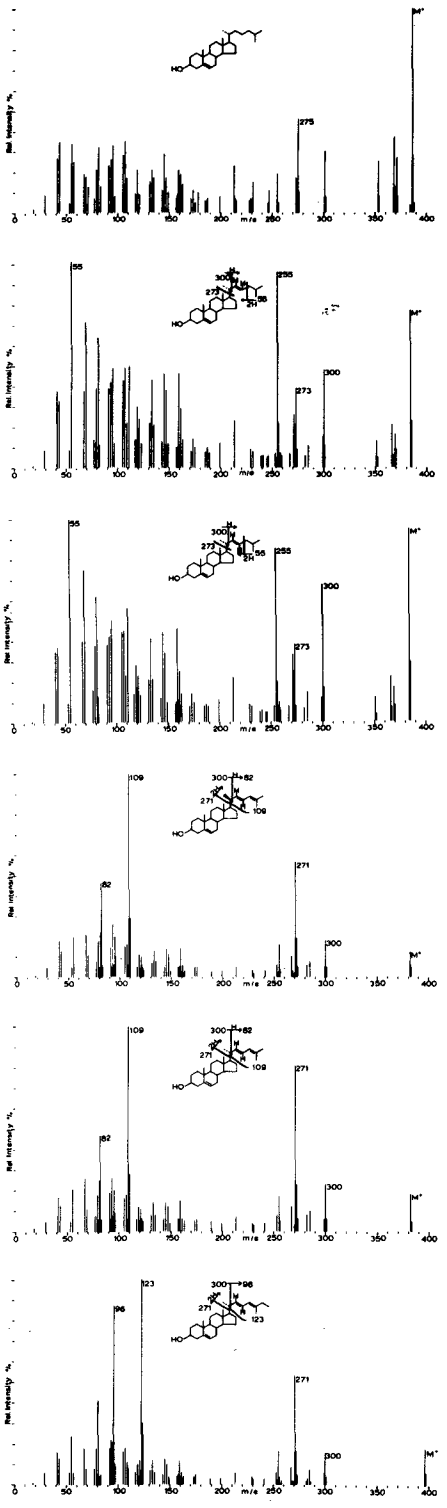

FIG. 2. Mass spectra of Δ^5-sterols with saturated, mono- and diunsaturated side chains.

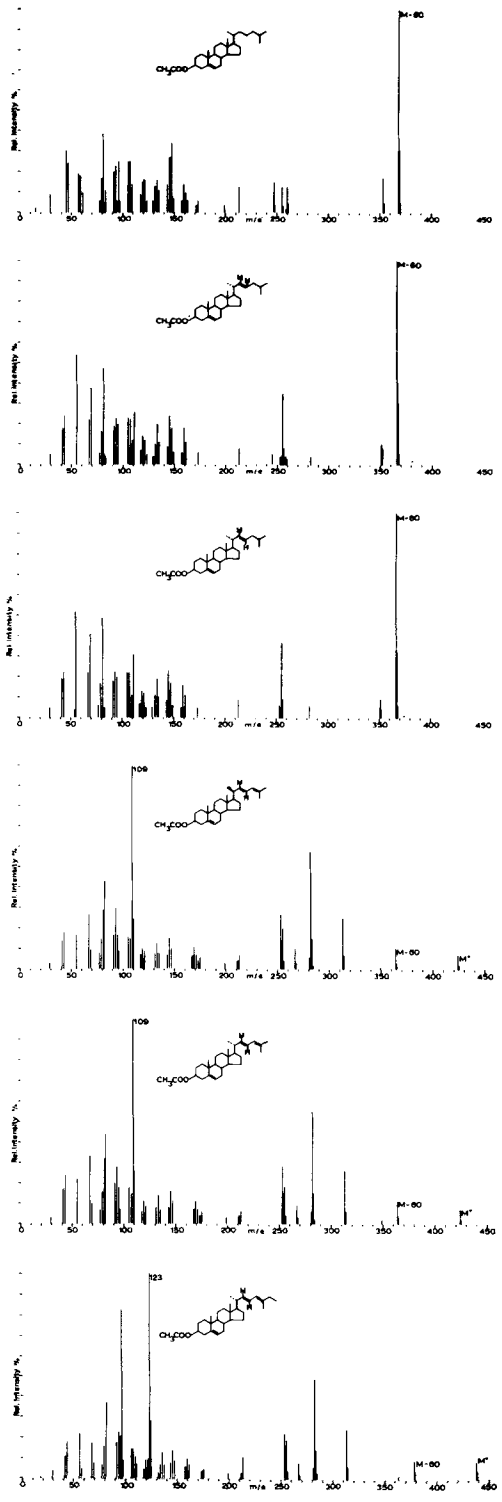

FIG. 3. Mass spectra of Δ^5-steryl acetates with saturated, mono- and diunsaturated side chains.

stigmasterol to cholesterol in the tobacco hornworm, E-22-cholesta-5,22,24-trien-3β-ol was isolated and identified as an intermediate (19). In order to confirm that this compound was indeed metabolized to cholesterol in the tobacco hornworm, it was necessary to synthesize E-22-cholesta-5,22,24-trien-3β-ol and the E and Z-22-dehydrocholesterols that could also be intermediates (25). MS of these sterols (Fig. 2) and their steryl acetates (Fig. 3) further demonstrate the usefulness of MS. All of the free sterols show strong M^+ peaks, except in the spectra of the sterols with a conjugated diene in the side chain. The Δ^5- and $\Delta^{5,22}$-sterol acetates parent ions were almost nonexistent. However, MS of the Δ^5-steryl acetates with the conjugated diene system in the side chain exhibited an M^+ and an M-60 peak that was only 10% of the base peak at m/e 109 and m/e 123 for the respective side chain, indicating that the conjugated double bond system stabilized the side chain moiety (25). Certainly, the fragmentation patterns give considerable information about the structure of these sterols.

The mass spectrum of 24-methylpollinastanol isolated from cultures of *C. sorokiniana* is shown in Figure 4. Compounds that have a 14-methyl group usually show a very strong M-15 peak as indicated by the peak at m/e 399, and the spectrum agreed with that of authentic 24-methylpollinastanol. Using argentation chromatography, GLC, GC-MS, derivatization and/or synthesis we have identified a number of new sterols from algae for which we reported MS data (14,26). There are also a number of papers that deal specifically with mass spectral analyses of sterols and their fragmentation processes (27,28).

The mass spectra of the ecdysteroids are characterized by numerous ions, 18 mass units

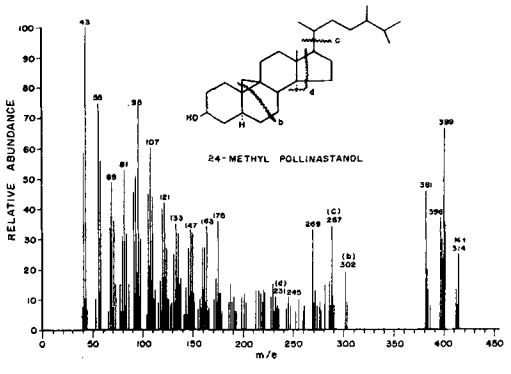

FIG. 4. Mass spectrum of 24-methylpollinastanol from *C. sorokiniana*.

apart, that result from successive losses of molecules of water from the molecule. Even though it is often difficult to obtain the molecular ions, the mass spectra thus provide valuable information about the molecular weight and the number of hydroxyl groups present in the molecule.

In an ecdysteroid such as 20-hydroxyecdysone that contains a 20,22-diol function, side-chain cleavage generally occurs at this location, as indicated in the spectrum by prominent peaks at m/e 363 (M-117) and 345 (M-117-18). This side-chain fragment is further indicated by prominent major peaks at m/e 99 and 81. Thus, mass spectral analyses can reveal considerable information about the ecdysteroid nucleus, side chain and molecular structure.

Proton Magnetic Resonance Spectra

The PMR spectra of steroids are generally complex because of the presence of a large number of aliphatic and alicyclic methylene groups. Nevertheless, studies of the PMR spectra of steroids have shown that the 3 proton signals of the methyl groups resulting from the equivalence of the protons were the most pronounced and sharpest peaks in the spectra, being above the background of methylene and methine protons in the region of δ 0.5-1.5 (29). Even in the spectra of steroids with methyl or ethyl groups in their side chain, it was possible to identify each methyl or ethyl substituent of the steroid (30). It was shown that substituents as well as stereochemical and conformational changes can have a pronounced effect on the chemical shifts of these methyl groups (30), thus demonstrating the practical application of PMR to structural problems of steroids. Tables of effects of substituents on the chemical shift of C-18 and C-19 protons have been compiled (31) and are most valuable for determining the position and orientation of functional groups in the steroid nucleus.

Spectra of sterols accumulated in our laboratory and published PMR data have served to establish the identity of sterols. The location of the C-18 and C-19 methyl groups was shown to be highly sensitive to the stereochemistry of the steroid nucleus and to the nature and orientation of the functional groups in the steroid nucleus or side chain. The side-chain methyls were simiarly influenced by substituents or functional groups in the side chain.

PMR has served us well in the determination of configuration at C-24, and the position of hydroxyl groups of ecdysteroids. The PMR spectrum of the Z-22-dehydrocholesterol shows that the C-18 methyl appears further downfield at δ 0.733 than the E-isomer C-18 methyl (0.700), a discernable difference of only 2Hz (25). Nevertheless, that difference was enough for the detection of the presence of 5% of E-isomer in the Z-compound and this can be used as a diagnostic tool to distinguish between the 2 C-22 isomers. Similar differences in the C-18 methyls were observed for the conjugated diene compounds, the structures of which are shown in Figure 2.

Although the chemical shift of the C-18 methyl does not distinguish the Z-Δ^{22}- from the Z-$\Delta^{22,24}$-compound or the E-Δ^{22}- from the E-$\Delta^{22,24}$-compound, the far downfield shift for the C-26 and C-27 methyl signal at δ 1.74 readily indicates a double bond at the C-24-position. For desmosterol, the C-26 and C-27 methyls appear as a doublet, centered at 1.67, indicating nonequivalence of the methyls in desmosterol (25,32).

A highly significant contribution of PMR to the identification of sterols was our finding that pairs of C-24 isomeric sterols could be distinguished from each other by PMR spectroscopy (33). We have observed that campesterol (24α-methylcholesterol) and 22,23-dihydrobrassicasterol (24β-methylcholesterol) even showed a difference in their spectra when recorded at 60 MHz, and the spectrum of the mixture definitely confirmed the difference (Fig. 5). For the sake of brevity, the spectra of the 3 other pairs of C-24 isomeric sterols that were studied (sitosterol and 22,23-dihydroporiferasterol, α-spinasterol and chondrillasterol, stigmasterol and poriferasterol) will not be presented or discussed.

The PMR spectra of campesterol and 22,23-dihydrobrassicasterol, recorded at 100 MHz, are quite different (Fig. 6). However, the spectrum of 22,23-dhydrobrassicasterol, prepared from ergoesterol (5), shows minor peaks in the region of 76.7 and 83.5 Hz, that appear in the spectrum of campesterol. These peaks were absent from the spectrum of 22,23-dihydrobrassicasterol, obtained from *Chlorella ellipsoidea* Gerneck (33), thus indicating that the synthetic 22,23-dihydrobrassicasterol contained some 24α-epimer. This finding suggested that during the hydrogenation of the Δ^{22}-bond in the preparation of 22,23-dihydrobrassicasterol (24β-methyl) some 24α-epimer was formed. We thought this to be unlikely. The spectra of our synthetic 22,23-dihydrobrassicasterol, campesterol and 22,23-dihydrobrassicasterol, obtained from *C. ellipsoidea* and recorded at 220 MHz, showed that our synthetic 22,23-dihydrobrassicasterol did indeed contain ca. 25% of the 24α-isomer (Fig. 7). In view of the recent findings (34) that tracheophytes ranging through the evolutionary hierarchy

from ferns through gymnosperms and primitive angiosperms to climax angiosperms consistently yield mixtures of 24-methylcholesterols (determined by PMR analyses), an alternative explanation for the origin of the 24α-epimer is that the ergosterol used in the synthesis of our 22,23-dihydrobrassicasterol consisted of a C-24

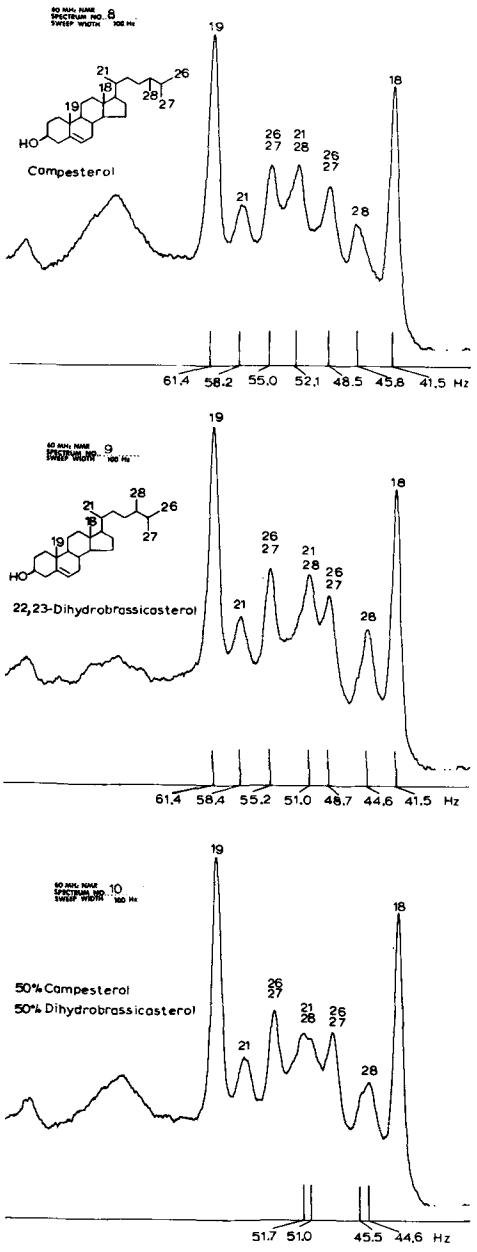

FIG. 5. Nuclear magnetic resonance spectra of campesterol and 22,23-dihydrobrassicasterol, recorded at 60 mHz.

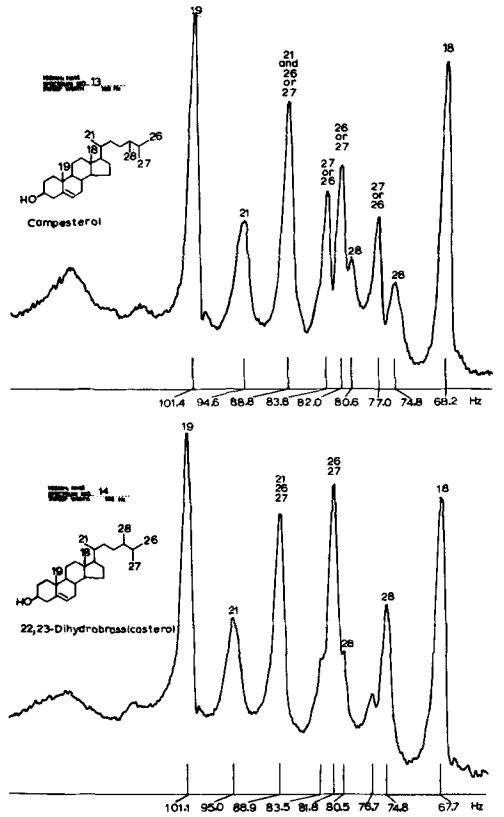

FIG. 6. Nuclear magnetic resonance spectra of campesterol and 22,23-dihydrobrassicasterol, recorded at 100 MHz.

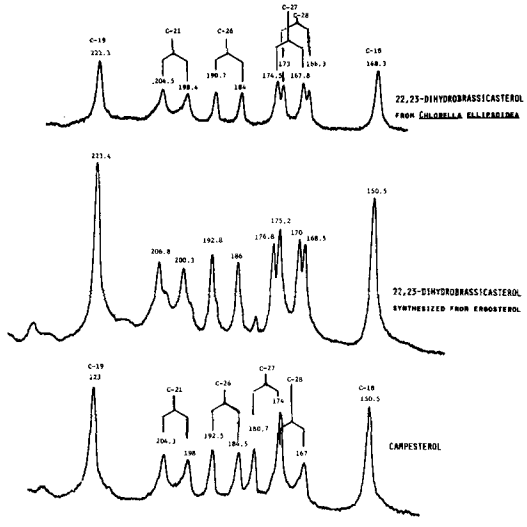

FIG. 7. Nuclear magnetic resonance spectra of campesterol and 22,23-dihydrobrassicasterol (synthetic and from *C. ellipsoidea*) recorded at 220 MHz.

epimeric mixture. Whether certain yeasts produce a C-24 epimeric mixture of ergosterol remains to be determined.

An extension of our studies through the use of a 220-MHz NMR spectrometer has made it possible to determine the configuration at C-24 of a large number of 24-methyl and 24-ethylcholesterols from tracheophytes (34). Thus, the sterols, 22,23-dihydroporiferasterol and sitosterol, that could not be distinguished from each other through their spectra recorded at 100 MHz, were readily distinguishable at 200 MHz. In fact, the presence of dihydrobrassicasterol, campesterol, or Δ^{22}-sterols as impurities can be detected in the spectra of either dihydroporiferasterol or sitosterol. The use of 220-MHz NMR spectrometry has permitted not only the characterization of the C-24 epimeric sterols of scallops but also a semiquantitative estimate of the 24α- and 24β-epimers present in the mixtures (35).

The PMR spectra of free ecdysteroids, usually obtained in deuteropyridine, are complex, but the resonance of the methyl groups of the ecdysteroids generally appear as sharp peaks in the spectra of these compounds and provide valuable information concerning their structures, especially with respect to the position of the various hydroxyl groups. Since the ecdysteroids of insects as well as many plant ecdysteroids differ from one another only by number and/or position of hydroxyl groups, all of the insect ecdysteroids and a large number of plant ecdysteroid structures (11) could be derived simply by MS analyses and a comparative examination of their PMR spectra with that of ecdysone, the structure of which has been established by X-ray diffraction (36).

The PMR spectral data recorded at 60 MHz for the ecdysteroids of the tobacco hornworm (Fig. 8) are presented in Table V. The spectrum of ecdysone shows a doublet for theC-21 methyl at δ 1.23 and 1.32 and peaks of equal intensity for the C-18 and C-19 methyls at δ 0.74 and 1.06, respectively, whereas the methyls at C-26 and C-27 appear also as a singlet and the strongest peak in the spectrum at δ 1.39. Mass spectral data for an ecdysteroid that was slightly more polar than ecdysone indicated that it contained one hydroxyl group more than ecdysone. The PMR spectra showed that all the methyl signals appeared as singlets, 3 of which were of equal intenstiy. There was no change in the position or degree of intensity for the C-19, C-26 and C-27 methyls, thus indicating that the environments for these methyls and for those in ecdysone are similar. The fact that the C-21 methyl appears as a singlet at δ 1.58 placed the additional hydroxyl group at C-20. This assignment was further supported by the shifting of the C-18 methyl downfield to δ 1.22. Thus, the ecdylsteroid was assigned the structure of 20-hydroxyecdysone.

Similarly, the position for a hydroxyl group was arrived at for an insect ecdysteroid that was more polar than 20-hydroxyecdysone. Its mass spectrum indicated that it contained one more hydroxyl group than 20-hydroxyecdysone. Its PMR spectrum showed that the 4 methyl resonances were of equal intensity, with 3 of them occurring at identical positions as the C-18, C-19 and C-21 methyls of 20-hydroxyecdysone; the additional hydroxyl group was placed at C-26. This compound was identified as 20,26-dihydroxyecdysone (Table V).

MS of the major ecdysteroid isolated from tobacco hornworm eggs indicated that its empirical formula was identical to that of 20-hydroxyecdysone. The PMR spectrum showed 3 methyl resonance peaks of equal intensity,

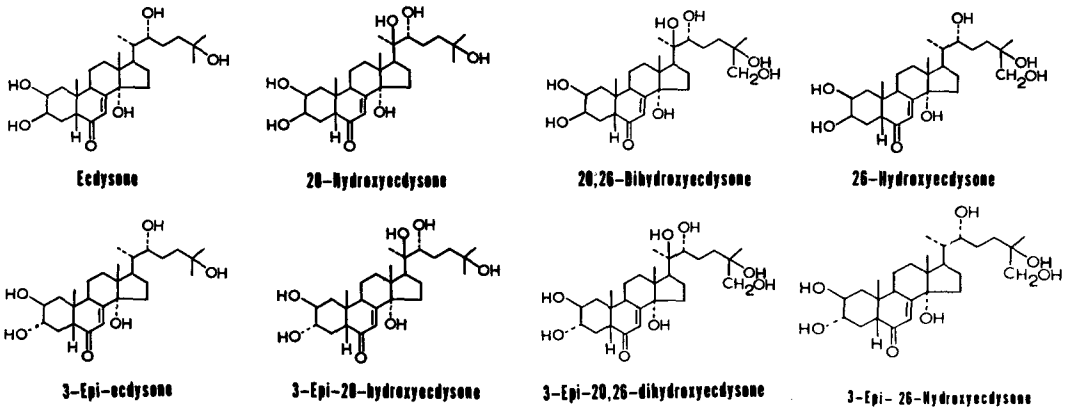

FIG. 8. Structures of ecdysteroids of the tobacco hornworm.

TABLE V

Nuclear Magnetic Resonances Absorption of Ecdysteroids Isolated from the Tobacco Hornworm

Ecdysteroids	Proton magnetic resonances/ methyl resonances (δ)			
	C-18	C-19	C-21	C-26- and/ or C-27
Ecdysone	0.74	1.06	1.23,1.32	1.39
3-*epi*-Ecdysone	0.75	1.07	1.23,1.32	1.40
20-Hydroxyecdysone	1.22	1.08	1.58	1.38
3-*epi*-20-Hydroxyecdysone	1.22	1.07	1.58	1.38
20,26-Dihydroxyecdysone	1.22	1.08	1.58	1.47
3-*epi*-20,26-Dihydroxyecdysone	1.22	1.07	1.58	1.48
26-Hydroxyecdysone	0.74	1.08	1.23,1.32	1.47
3-*epi*-26-Hydroxyecdysone	0.73	1.06	1.23,1.31	1.49

and it also showed that the C-18, C-19 and C-21 methyls occur in positions identical to those in ecdysone and the other methyl is at the same position as that of the C-27 methyl of 20,26-dihydroxyecdysone. This indicated that this ecdysteroid was 26-hydroxyecdysone.

For each of the 4 3β-ecdysteroids of the tobacco hornworm, we have isolated a correspondingly less polar compound (10,37). All the less polar ecdysteroids exhibited MS data and methyl resonances (Table V) that were almost indistinguishable from the respective more polar ecdysteroid. These characteristics and the fact that there was no noticeable downfield chemical shift in the C-19 methyl suggested that these less polar ecdysteroids were different in orientation of the hydroxyl group at C-3 from the respective corresponding more polar compounds. When these ecdysteroids were allowed to react with acetone, they either yielded no acetonide or one less acetonide than the corresponding 3β-epimer. Thus, they were identified as 3-epi-ecdysteroids (Fig. 8). It is possible that PMR spectra of the 3β- and 3α-ecdysteroids recorded at 220 MHz may show sufficient chemical shift differences in their C-19 methyls that would distinguish these compounds.

CONCLUSIONS

We have presented our methods of extraction, separation and isolation of steroids from insects and algae and have tried to show how the integration of various analytical instruments with proper isolation techniques can expedite the identification of steroids. Not every investigator may have use or immediate need for all of the tools we have employed in our sterol research, but s/he should be cognizant of what instruments are available and how s/he can best use them for maximal results. What we have presented here indicates only a fraction of potential uses of these instruments. Even with all of our modern instruments, for the chemist, th final proof of structure of a compound that has not been previously identified is its synthesis. We have synthesized a number of steroids that were isolated in our laboratory as well as compounds that may serve as molecular models for those that are more difficult to achieve by synthesis.

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The Determination of Steroids with and without Natural Electrophores by Gas Chromatography and Electron-capture Detection

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ABSTRACT

The response of the electron-capture detector to organic compounds is poorly defined, and the steroids are no exception to this observation. For those steroids which are naturally electron-capturing, the structures of the electrophores will be defined. Other steroids can be made electron-capturing by the formation of appropriate derivatives. Some new or infrequently used reagents for this purpose (flophemesyl ethers, *t*-buflophemesyl ethers, pentafluorophenylhydrazone derivatives and halogen-substituted aromatic boronic acids) are described.

INTRODUCTION

The impact of gas chraomtography (GLC) in the area of biochemical analysis has been enormous. This is no less true for steroid analysis, even though these substances are often thermally labile. A perusal through the early literature of gas chromatography shows that many workers in the steroid field made significant contributions to the improvement of column technology, liquid phases, selective detectors and, particularly, to the technique of derivatization. As a consequence of this effort, the production of steroid profiles from complex biological fluids can be realized in many laboratories today. This has had impact on the recognition and diagnosis of disorders of the body associated with steroid hormonal dysfunction.

The electron-capture detector (ECD) is the most sensitive of the gas chromatography detectors in routine use. It is a selective detector with a very broad range of response (covering 7 orders of magnitude) for organic compounds, depending on their elemental composition and molecular structure. Because of its ability to detect picogram (10^{-12} g) quantities of steroids under favorable conditions, it has been much used for this purpose—although few steroid chemists are familiar with its response/molecular structure relationship. As only a few steroids show a large response to the electron-capture detector, it would be of little interest to the steroid analyst if it were not for the technique of derivatization. Many steroids are thermally labile and must be derivatized prior to analysis to avoid decomposition of the compound and to improve its chromatographic performance. Derivatizing reagents are available which contain an electron-capturing group and can be used to attack this tag to the steroid and at the same time meet the requirements of improving thermal stability and chromatographic performance. Using this technique, nearly any steroid can be made electron-capturing and some of the methods available will be described in this paper.

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EXPERIMENTAL

The flophemesyl, *t*-buflophemesyl, 2,4-dichlorobenzeneboronic acid and 3,5-*bis*-(trifluoromethyl)benzeneboronic acid reagents were obtained from Lancaster Synthesis (St. Leonard Gate, Lancaster, Great Britain) and the Alfa Products Division, Ventron Corporation (Danvers, MA). Pentafluorophenylhydrazine was obtained from Aldrich Chemical Company (Milwaukee, WI) and was purified as follows: the crude sample was treated with charcoal and recrystallized 3 times from light petroleum (bp 100-120 C), followed by chromatography on Davison silica gel eluted with chloroform. The reagent was further purified by conversion to its hydrochloride, followed by recrystallization from ether/ethanol and sublimation to constant mp 239-242 C.

RESULTS AND DISCUSSION

A discussion of the physical basis of the operation of the electron-capture detector is beyond the scope of this paper and has been reviewed previously (1-4). In this paper, we will concentrate on a discussion of structure-response relationships of steroids and their derivatives with thermal electrons produced in the ECD.

TABLE I

The Relative Molar Response of Some Representative Steroids toward the ECD

Steroid	Relative Molar Response
Androstane	1
Cholestane	1
Cholesterol	3
25-Keto-*nor*-cholesterol	3
4,4'-Dimethyl-5-cholest-ene-3-one	3
Estrone	3
Androstane-3,17-dione	10
1-Androst-ene-3,17-dione	21
Allopregnane-3,11,20-trione	50
1,4-Androst-ene-3,17-dione	450
i-Cholestan-6-one	580
Testosterone	840
17β-Hydroxy-1,4,6-androst-triene-3-one	850
19-Nortestosterone	910
4-Cholest-en-3-one	1130
4-Androst-ene-3,17-dione	1920
Progesterone	2220
4-Androst-ene-3,16-dione	2350
11β-Hydroxy-4-androst-ene-3,17-dione	7600
3,5-Cholest-diene-7-one	9650
6-Ketoprogesterone	14300
1,4,6-Androst-triene-3,17-dione	16700
4-Androst-ene-3,11,17-trione	24800
4-Pregn-ene-3,11,20-trione	25000
1,4-Androst-diene-3,11,17-trione	53500

Steroids with Natural Electrophores

Organic compounds in general show a very wide range of response to the ECD. The steroids as a class of organic compounds are no exception to this observation. The relative molar response of some representative steroids is shown in Table I (5). The steroid hydrocarbons and steroids containing various numbers of hydroxyl groups are virtually transparent to thermal electrons and cannot be determined at low concentrations with this detector. On the other hand, steroids containing α,β-unsaturated ketone groups produce a high response, the magnitude of which is influenced by the substituents attached to the double bond of the unsaturated ketone group. The highest response is obtained with steroids having substituents capable of assisting in stabilizing or delocalizing the captured electron. The interactions between the unsaturated ketone group and the substituent groups may be quite subtle and can take place across or through several saturated carbon-to-carbon bonds. Geometric position would seem to be a very important parameter. For example, 4-androst-ene-3,17-dione is ca. 100 times more sensitive than 1-androst-ene-3,17-dione. Likewise, for those steroids with a 4-en-3-one group, the detector response is little affected by the position of the ketone group in the D ring but the introduction of a ketone group at the C-11 position can increase the detector sensitivity by more than 3 orders of magnitude.

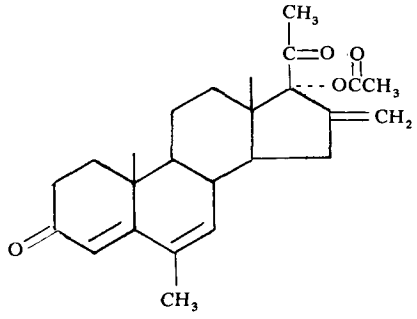
Isolated ketone groups in a saturated steroid framework confer little electron-capturing ability on the molecule unless conjugated with a double bond. All the very strongly electron-capturing steroids identified to date contain a minimum of an α,β-unsaturated ketone group, conjugated in some way with other functional groups in the steroid framework.

Advantage can be taken of the natural electron-capturing properties of steroids to enable them to be determined at trace levels. When no hydroxylic groups are present and the steroids are thermally stable, derivatization prior to analysis is not required. An example of this is the determination of melengestrol acetate, which can be determined in tissue extracts at the parts per billion (ppb) level (detection limit 2-25 ppb) (6-8).

Melengestrol Acetate

Melengestrol acetate (MGA, 17α-acetoxy-6-methyl-16-methylene-4,6-pregna-diene-3,20-dione, Scheme I) is incorporated into animal feed as an oral progestational agent to inhibit ovulation and suppress estrus. It is also effective as a growth-promoting agent, giving a higher conversion of feed to meat. Because of the strong physiological effect of this hormone similar to that of the contraceptive hormones,

residues of this steroid in meat must be low at the time of marketing.



SCHEME I. Melengestrol acetate.

Koshy (9) has studied a series of steroids related to melengestrol acetate to better identify the molecular features important for a high ECD response. The relative molar response of the model steroids towards the ECD are summarized in Table II. From their data, the following conclusions can be drawn: (a) the 17α-acetate substituent is an important part of the electrophore, as progesterone itself had only a low response to the ECD; (b) the 17α-propionate had a similar response to that of the acetate; (c) methyl substituents in rings A or B at C-2, C-6, or C-7 did not effect the detector response; (d) for the unsaturated ketone systems, the order of sensitivity was 4,6-dien-3-one > 1,4-dien-3-one > 4,9(11)-dien-3-one > 4-en-3-one; (e) a methyl or methylene substituent at C-16 gives an increase in detector response, a methylene substituent being more effective than a methyl substituent; (f) a large increase in detector response for the 4-en-3-one system was obtained by introducing a ketone group at C-6.

Thus, in the case of melengestrol acetate, the important components of the electrophore can be identified as the 4-en-3-one system of ring A, the 6-double bond of ring B, the C-16 methylene unit attached to ring D and the 17α-acetoxy group attached to ring D. The on-column detection limit for this compound was ca. 10-20 picograms.

Ecdysones

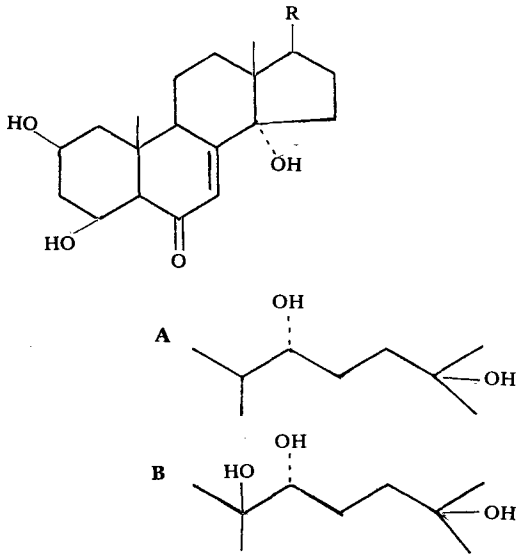
Some steroids containing a natural electrophore are thermally labile and as such can not be determined without derivatization. In this case, the derivatization reaction does not enhance detection, but is used simply to improve the thermal or chromatographic properties of the steroid. Such a situation is found among the ecdysteroids (Scheme II), the insect-molting hormones.

The relative molar response of the ECD towards some ecdysone trimethylsilyl (TMS) derivatives and related steroids is given in Table III (10). The ketones 5α-cholestan-6-one and 5α-cholest-7-en-6-one, show no special selectivity toward the ECD. The introduction of a substituent at C-14 leads to a marked increase in sensitivity (ca. 1,300-fold in the case of the C-14 TMS ether). The C-14 TMS ether is 21 times more sensitive than the free hydroxyl group in the model steroid 2β,3β,14α-trihy-

TABLE II

Relative Molar Response of the ECD to Some Model Steroids Related to Melengestrol Acetate

Steroid	Relative molar response
2α-Methyl-17α-acetoxyprogesterone	0.94
17α-Acetoxyprogesterone	1.00
6α-Methyl-17α-propoxyprogesterone	1.10
17α-Propoxyprogesterone	1.14
6α-Methyl-17α-acetoxyprogesterone	1.30
2α-Methyl-17α-acetoxy-4,9-pregn-diene-3,20-dione	1.40
6α-Methyl-17α-acetoxy-4,9-pregn-diene-3,20-dione	1.40
17α-Acetoxy-1,4-pregn-diene-3,20-dione	1.90
6α-Methyl-17α-acetoxy-1,4-pregn-diene-3,20-dione	1.90
17β-Acetoxy-4,6-pregn-diene-3,20-dione	5.60
17α-Acetoxy-4,6-pregn-diene-3,20-dione	7.30
6β-Methyl-17α-acetoxy-4,6-pregn-diene-3,20-dione	7.30
6α,7α-Dimethyl-16-methylene-17α-acetoxy-4-pregn-ene-3,20-dione	12.90
16-Methylene-17α-acetoxy-4-pregn-ene-3,20-dione	13.50
6α-Methyl-16-methylene-17α-acetoxy-4-pregn-ene-3,20-dione	14.80
1,4-Androst-diene-3,11,17-trione	16.90
6β,16α-Dimethyl-17α-acetoxy-4-pregn-ene-3,20-dione	22.90
17α-Acetoxy-4-pregn-ene-3,6-20-trione	25.00
6β-Methyl-16-methylene-17α-acetoxy-4,6-pregn-diene-3,20-dione	45.80



SCHEME II. (A) Ecdysone, (B) ecdysterone.

droxy-5α-cholest-7-en-6-one. However, silylation of the C-14 hydroxyl group can affect the response of the ECD in an unpredictable manner (Table IV). The 2β,3β-*bis*-TMS-5α(β)-cholest-7-en-6-ones are 40 times more sensitive than the unsaturated ketone, although the 2 TMS groups are remote from this center. As both the 5α- and 5β-epimers are equally sensitive, the geometry of the A, B ring junction is not important. The 2β,3β,14α-*tris*-TMS-5α(β)-cholest-7-en-6-ones are some 6 times more sensitive to detection than the 14α-TMS-5α-cholest-7-en-6-one and, thus, the remote 2β,3β-TMS groups make a small contribution to the electrophore. The electrophore in the ecdysteroids can be identified as consisting of the 7-en-6-one group and the substituent attached to C-2 and C-3. The TMS derivative of the ecdysones can be detected with an ECD at low picogram levels in insect and crustacean samples (11-13).

Derivatization As a Technique of Introducing an Electrophore into Steroids

For the detection of steroids with little or no native electron-capturing properties, an electrophore can be introduced by the technique of derivatization. Steroids with polar functional groups such as hydroxyl, phenolic, or carboxyl groups, often require prior derivatization to improve their thermal stability and chromatographic performance. In such cases, derivatization is not an extra chemical step but an integral part of the analytical scheme. A reagent is selected which contains a strongly electron-capturing group and this is made to react with the polar functional group of the steroid to produce a derivative which is volatile, thermally stable and, of course, sensitive to the ECD. As the molecular weight of steroids is quite high (as far as GLC is concerned), the reagents employed for their derivatization have either a low molecular weight or good volatility characteristics (e.g., polyfluorinated compounds), so as not to have an adverse effect on the volatility of the steroid. As for reagents for use with the ECD, the halocarbonacylating compounds (e.g., heptafluorobutyryl, pentafluoropropyl, chlorodifluoroacyl) are the most widely used derivatives. It would be inappropriate in this paper to attempt a detailed review of the available literature in this area. The

TABLE III

Relative Molar Response of Some TMS Derivatives of Ecdysone and Related Steroids

Steroid	Minimum detectable quantity ($\times 10^{-9}$ g)	Relative molar response
TMS-Cholesterol	200	1.0
5α-Cholestan-6-one	40.0	5.0
5α-Cholest-7-en-6-one	40.0	5.0
17β-TMS-17α-Methyltestosterone	5.0	40.0
4-Cholest-ene-3,6-dione	5.0	40.0
2β,3β-Di-TMS-5α-cholest-7-en-6-one	1.0	200
5α-Acetoxy-7-cholest-en-6-one	0.25	800
14α-Hydroxy-5α-cholest-7-en-6-one	0.07	2857
2β,3β-Di-TMS-14α-hydroxy-5α-cholest-7-en-6-one	0.005	4000
14α-TMS-5α-cholest-7-en-6-one	0.03	6667
2β,3β,14α-Tri-TMS-5β-pregn-7-ene-6,20-dione	0.005	40,000
2β,3β,14α-Tri-TMS-5α-cholest-7-en-6-one	0.005	40,000
penta-TMS-ecdysone	0.005	40,000
hexa-TMS-ecdysterone	0.005	40,000
hexa-TMS-inokosterone	0.005	40,000
penta-TMS-cyasterone	0.005	40,000

TABLE IV

Relative Molar Response of the ECD to Ecdysone Analogs with and without Silyation at C-14

Steroid	Response ratio 14α-TMS/14α-OH
Ecdysone	0.3
Ecdysterone	0.9
2-Deoxy-20-hydroxyecdysone	1.1
Inokosterone	0.5
Poststerone	0.3
2β,3β,14α-Trihydroxy-5α(β)-cholest-7-en-6-one	12.0

methods for formation and properties of nearly all the derivatives of the most studied steroids can be found in 2 recently published handbooks on derivatization (14,15).

In this paper we will review some of the recent work in progress in our laboratories and how it relates to the formation of steroid derivatives for use with the electron-capture detector.

Steroid Flophemesyl Derivatives

Perhaps the most common and universal derivatization reaction for use in GLC is the formation of the TMS ethers and esters (16). This is the most frequently used reaction for forming steroid derivatives, but as it neither introduces an electron-capturing group into the steroid nor improves detection, unless a silicon-specific detector is available, this reaction is not especially useful for trace analysis. The replacement of one of the methyl units of the TMS group by a halocarbon substituent should maintain the useful volatility and reactivity of the TMS reagents while providing high sensitivity to the ECD. A compromise has to be struck between the volatility of the derivatives and their ECD sensitivity. This is apparent from the data given in Table V for some halocarbonsilyl ether derivatives of cholesterol. In terms of ECD sensitivity, the order of response is I > Br > Cl >> F, which is the reverse order of the volatility of the derivatives in GLC. Thus, although iodine and bromine are more sensitive to the ECD than fluorine, their greater mass produces less volatile derivatives, which limits their use in steroid analysis. Closely bound fluorine atoms in an alkyl or aryl compound are remarkable in that they show very little increase in boiling-point compared to hydrocarbons containing a similar number of carbon atoms (the increase in molecular weight being offset by a decrease in intermolecular bonding forces in the fluorocarbon). It was for this reason that fluorocarbon substituents were selected to make new derivatizing reagents for steroid analysis (17, 18). The high volatility of fluorine compounds enables multiple substitution of fluorine into the reagent without a large change in volatility (Table V) and also favors an increase in ECD sensitivity, especially if the captured electron can be stabilized by delocalization. The heptafluorobutyryl ester derivatives owe much of their popularity in steroid analysis to the

TABLE V

Relative Volatility and ECD Sensitivity of a Series of $RR_1Si(CH_3)$-Cholesterol Ethers

R	R_1	Relative retention time[a]	Minimum detectable quantity ($\times 10^{-9}$)
CH_3	CH_3	1.0	
$CF_3(CH_2)_2$	CH_3	1.26	1,500
$CF_3(CF_2)_2(CH_2)_2$	CH_3	1.37	115
$ClCH_2$	CH_3	2.10	75
C_6F_5	CH_3	3.14	4
$C_6F_5(CH_2)_2$	CH_3		200
CH_2Br	CH_3	5.13	0.5
C_6F_5	CH_2Cl	6.26	
C_6F_5	$C(CH_3)_3$	6.30	
CH_2I	CH_3	12.82	0.005

[a]Column of 1% OV-101 on Gas Chrom Q 100-120 mesh; nitrogen flow rate, 75 ml min^{-1}.

volatility conferred by the perfluorocarbon group and the presence of the conjugated carbonyl group, which helps to stabilize the captured electron. The perfluoroalkane groups used here do not have any possibility to stabilize the negative charge formed upon electron capture (dissociation of the CF bond is unlikely on thermodynamic grounds), and this results in a rapid elimination of the captured electron. The perfluoroalkane-containing silyl ethers lack the required ECD sensitivity for trace analysis. On the other hand, the pentafluorophenyldimethylsilyl (flophemesyl) ethers show remarkable ECD sensitivity, being 20 times more sensitive than the chloromethyldimethylsilyl ether. It is interesting that the pentafluorophenyl group, when separated from silicon by an alkyl chain as in the 2′-pentafluorophenylethyldimethylsilyl ether, is 50 times less sensitive to the ECD than the flophemesyl derivative. A possible explanation for this is that in the flophemesyl ether the captured electron is buried in the π-orbitals of the phenyl ring, which is further stabilized by ($p \rightarrow d$) π-bonding with the low-energy *d*-orbitals of silicon. In the case of the pentafluorophenylethyldimethylsilyl ether, back-bonding of the type just described does not occur.

For the analysis of steroids at low levels, in which both volatility and sensitivity to the ECD are important, the flophemesyl derivatives are the most useful (17-19). In pyridine solvent, the reactivity of the flophemesyl reagents toward steroid hydroxyl groups is: flophemesylamine > flophemesyl chloride > flophemesyldiethylamine > flophemesyldisilazane >> flophemesylimidazole. The uncatalyzed reactions of flophemesyldisilazane and flophemesyldiethylamine with cholesterol parallel those of the trimethylsilyl reagents, giving 85% conversion in 24 hr at room temperature. Increasing the reaction temperature does not improve the yield. Flophemesyl chloride in chloroform reacts with cholesterol to form ca. 80% of the flophemesyl derivative after heating at 60 C for 16 hr, whereas in pyridine the reaction goes to completion in 2-3 hr. In the absence of an acid acceptor like pyridine, flophemesyl chloride is not a strong flophemesylating reagent. Flophemesylamine reacts quantitatively with cholesterol and other steroids with unhindered secondary hydroxyl groups in a variety of solvents at room temperature. It does not react with tertiary hydroxyl groups, such as the 17β-OH in 17α-methyl-17β-hydroxy-4-androsten-3-one or with hindered secondary hydroxyl groups such as the 11β-OH in 11β-hydroxy-4-androst-ene-3,20-dione. As an added advantage, it does not promote the formation of flophemesyl enol ethers from unprotected ketone groups. Flophmesylamine at ambient and elevated temperatures does not react with ketone groups at C-3, C-6, C-11, C-17, and 4-en-6-one or 7-en-6-one groups. It is thus the reagent of choice for the formation of flophemesyl ethers of unhindered secondary hydroxyl groups. It also enables a distinction to be made between normal secondary hydroxyl groups and hindered and tertiary hydroxyl groups, with which it is totally unreactive.

The addition of an acid catalyst to a flophemesyl reagent increases its reactivity, reducing the time required for complete reaction and causing hindered hydroxyl groups to react as well. This is not particularly marked with the flophemesylamine reagent, and since other flophemesyl reagents show a marked increase in activity with acid catalysis, the amine is more useful as an uncatalyzed, selective protecting reagent.

All the Lewis-type acid catalysts tried have catalytic properties, but the most efficient catalyst is flophemesyl chloride or flophemesyl bromide. Boron trifluoride and *p*-toluenesulfonyl chloride or acid have a advantages in being soluble in organic solvents, but small amounts of secondary peaks from decomposition of the steroid are observed. Aluminum chloride is exceptional in producing a large number of secondary peaks. Ammonium sulfate is inefficient, probably because of its low solubility in the reaction mixture.

Using flophemesyl chloride as catalyst, the reactivity of the flophemesyldisilazane and particularly the flophemesyldiethylamine increases markedly. The catalyzed flophemesyldisilazane gives a quantitative yield of flophemesyl cholesterol in 3 hr at 65 C and the flophemesyldiethylamine in less than 15 min at room temperature. The flophemesyldisilazane does not react with hindered secondary hydroxyl groups, even with a catalyst.

Flophemesyldiethylamine, when catalyzed by flophemesyl chloride in pyridine as solvent, was the strongest flophemesylating reagent mixture found. The reactivity of the reagent combination depends on the amount of catalyst employed. A 10:1 mixture of flophemesyldiethylamine and flophemesyl chloride reacts quantitatively with unhindered secondary hydroxyl groups of the cholesterol type and also tertiary hydroxyl groups, such as the 17β-OH of 17α-methyl-17β-hydroxy-4-androst-ene-3-one. It does not react with the hindered 11β-OH in 11β-hydroxy-4-androst-ene-3,17-dione. Unprotected ketone groups are not enolized by this reagent combination. A 1:1 mixture of flophemesyldiethylamine and flophemesyl

TABLE VI

Conditions for the Formation of Flophemesyl Steroid Ethers

Steroid	Flophemesyl chloride in pyridine	Flophemesyl-amine	Flophemesyl-diethylamine: flophemesyl chloride 10:1	Flophemesyl-diethylamine: flophemesyl chloride 1:1
Cholesterol	A	B	B	B
Ergosterol	A	B	B	B
Cholestanol	A	B	B	B
2β,3β-Dihydroxy-5α-cholestane	A	B	B	B
2β,5α,6β-Trihydroxycholestane	2β,6β A	2β,6β B	2β,6β B	2β,6β B
2β,5α-Dihydroxycholestan-6-one	2β A[a]	2β B	2β B	NQ[a]
2β,3β,14α-Trihydroxy-7-cholest-en-6-one	2β,3β A[a]	2β,3β B	2β,3β B	2β,3β B[a]
3α,20α-Dihydroxy-5β-pregnane	A	B	B	B
17α-Methyl-17β-hydroxy-4-androst-en-3-one	NQ[a]	NR	B	B[a]
11β-Hydroxy-4-androst-ene-3,17-dione	NR	NR	NR	A
17α-Hydroxy-4-pregn-ene-3,20-dione	NR	NR	NR	C
3α,17α,20α-Trihydroxy-5β-pregnane	CP	CP	CP	CP
17α,21-Dihydroxy-4-pregn-ene-3,11,20-trione	IR	IR	IR	-
17α,11β,21-Trihydroxy-4-pregn-ene-3,20-dione	IR	IR	IR	NQ

A = 3 hr at 60 C; B = 0.25 hr at room temp; C = 6 hr at 85 C; NR = no reaction; NQ = none quantitative; CP = cyclic product; IR = not all hydroxyl groups react.
[a]Ketone protected as its methoxime.

TABLE VII

Reactivity of Flophemesyl Reagents

Reagent	Hydroxyl group environment
Flophemesyl chloride in pyridine	Unhindered secondary hydroxyl groups[a] when ketones are first protected.
Flophemesylamine	Selectively reacts with unhindered secondary hydroxyl groups[b] in the presence of ketone groups. Does not react with tertiary or hindered secondary hydroxyl groups.
Flophemesyldiethylamine flophemesyl chloride (10:1)	Unhindered secondary hydroxyl groups and exposed tertiary hydroxyl groups[c] in the presence of ketones.
Flophemesyldiethylamine flophemesyl chloride (1:1)	Unhindered and hindered secondary, and exposed tertiary hydroxyl groups. Very hindered tertiary hydroxyl groups[d] do not react completely. Ketone groups must be protected.

[a]The 3β-OH of cholesterol is taken to be a typical unhindered secondary hydroxyl group.

[b]The 11β-OH of 11β-hydroxy-4-androst-ene-3,17-dione is taken to be a typical hindered secondary hydroxyl group.

[c]The 17β-OH of 17α-methyl-17β-hydroxy-4-androst-en-3-one is taken to be a typical exposed tertiary hydroxyl group.

[d]The 17α-OH of 17α,11β,21-trihydroxy-4-pregn-ene-3,20-dione is taken to be a typical very hindered tertiary hydroxyl group.

chloride reacts readily with ketone groups, necessitating their protection, usually as the methoxime derivative, before reaction of the hydroxyl groups. In pyridine, this reagent combination completely converts the 11β-OH of 11β-hydroxy-4-androst-3,17-dimethoxime to its flophemesyl ether in 3 hr at 60 C and the 17α-OH of 17α-hydroxy-4-pregn-ene-3,20-dimethoxime to the flophemesyl ether in 6 hr at 85 C or 1 hr at 150 C. The 14α-OH of 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-methoxime is unaffected by these reagents. The reactivity of the flophemesyl reagents with various steroids is summarized in Tables VI and VII.

The pentafluorophenyl ring is a strongly electron-attracting group which is able to influence the mode of fragmentation of steroid derivatives under electron impact in a way which leads to diagnostic mass spectra often showing marked differences from those of the TMS ethers. The flophemesyl derivatives generally show a strong molecular ion with enhanced steroid hydrocarbon fragments as less of the total ion current is carried by the silicon-containing fragments (e.g., the mass spectra of the TMS [Figure 1] compared to the flophemesyl derivative [Figure 2] of 17α-methyl-17β-hydroxy-4-androst-ene-3-one). The flophemesyl derivative provides much more detailed diagnostic information about the structure of the parent steroid than does the TMS derivative.

In an attempt to prepare a reagent with similar ECD properties but greater hydrolytic stability than the flophemesyl reagents, we have

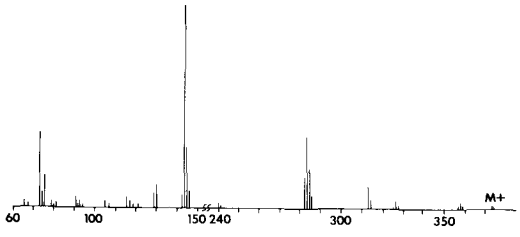


FIG. 1. Electron-impact mass spectrum at 70 eV of 17α-methyl-17β-4-hydroxyandrost-en-3-one TMS ether.

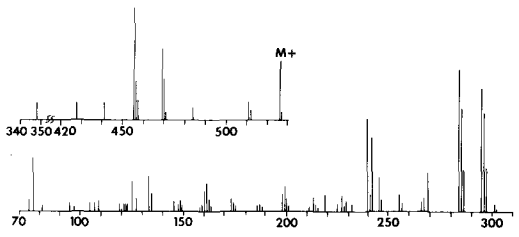


FIG. 2. Electron-impact mass spectrum at 70 eV of 17α-methyl-17β-4-hydroxyandrost-en-3-one flophemesyl ether.

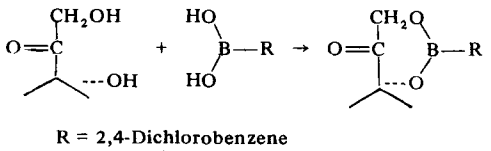
prepared *t*-butylpentafluorophenylmethylchlorosilane (*t*-buflophemesyl chloride) and used this for the trace level analysis of alcohols (20). This reagent, in the presence of diethylamine as catalyst, reacted quantitatively at room temperature with unhindered secondary hydroxyl groups and with the tertiary 17β-OH group in 17α-methyl-17β-hydroxy-5α-androstane-3-one at 60 C for 3 hr. It did not react with the 11β-OH group in 11β-hydroxy-4-androst-ene-3,17-dione dimethoxime or with any more hindered hydroxyl groups.

Selective Chemical Reactions for the Introduction of Electrophores into Steroids

Selective chemical reactions are useful when it is required to obtain some precise quantitative data of relatively few components in a complex mixture. If one can combine the chemical specificity of the derivatization reaction with a detector which is both selective for the reagent and has a high response to the derivative, then a simple form of analysis without excessive preliminary chromatographic isolation and concentration becomes possible. For this purpose we have investigated 2 reactions with the ECD (a) the formation of pentafluorophenylhydrazones of steroid ketone groups:

$$C{=}O + H_2NNHC_6F_5 \rightarrow \quad C{=}NNHC_6F_5 \ ,$$

and (b) the formation of electron-capturing boronates of diol groups:



R = 2,4-Dichlorobenzene
= 3,5-*bis*(trifluoromethyl)benzene

Pentafluorophenylhydrazones have been prepared and used in the analysis of estrone and estradiol (after oxidation) by GLC-ECD at levels down to 0.08 ng (21,22). The reagent for derivatizing the estrogens consisted of a solution of pentafluorophenylhydrazine in methanol/acetic acid (9:1). This reagent did not give a quantitative reaction with 5α-cholestan-3-one, 5α-cholestan-6-one and 4-cholest-ene-3,6-dione, as witnessed by unreacted steroid, which was identified in all cases. The optimal conditions for the formation of 2,4-dinitrophenylhydrazone derivatives of steroids have been identified as acid catalysis in polar solvents (23,24). Using ethanol as solvent, hydrochloric acid is the best catalyst as indicated by complete reaction of 5α-cholestan-3-one, determined by GLC and TLC. The reaction with acetic acid, phosphoric acid and oxalic acid in ethanol, dimethyl sulfoxide, dimethylformamide, tetrahydrofuran and dioxane was not quantitative, as witnessed by a peak for the ketone in GLC.

The product of the reaction between 5α-cholestan-3-one and pentafluorophenylhydrazine contained a minimum of 4 components as shown by GLC with poor volatility on a column of 1% OV-101. The products formed by GLC did not arise by thermal decomposition, as separation of the reaction mixture by TLC on silica gel with 30% ethyl acetate/chloroform as the mobile phase showed a minimum of 4 components. After spraying with an acid solution of stannous chloride and heating at 110 C for 15 min. Spotting with authentic 5α-cholestan-3-one and developing with 1% methanol/chloroform at right angles to the first direction indicated the presence of the keto steroid in 2 of the original 4 spots. The pentafluorophenylhydrazine was suspected as being impure and was purified and converted to the hydrochloride.

The pentafluorophenylhydrazine hydrochloride was a useful reagent allowing quantitative reaction with 5α-cholestan-3-one in ethanol for 2 hr at 55 C. The reagent also was more stable as its hydrochloride and did not discolor with time. It also obviates the necessity of adding mineral acid to the reaction mixture, which would be an advantage when working with acid-labile material.

The formation of cholestan-3-one pentafluorophenylhydrazone on a preparative scale yielded an orange oil which failed to crystallize after removal of solvent. Chromatography of the oil on bentonite/Kieselguhr (4:1) or silica gel showed decomposition of the derivative, and all fractions that contained the derivative were contaminated with the keto steroid. On preparative layer plates, 2 closely migrating bands were removed. The crude material from each band had a similar mass spectrum and an ion expected for the molecular weight of the keto derivative. It is likely that these 2 components are *syn*- and *anti*-isomers of the keto derivative. If the derivatives were allowed to stand for some time at room temperature, their color darkened noticeably and GLC produced complex chromatograms. Overall, the poor volatility of the pentafluorophenylhydrazone derivatives on GLC, their limited hydrolytic stability and potential instability when exposed to the atmosphere and direct light do not make these very useful steroid derivatives. Koshy et al. (25) have prepared *O*-(2,3,4,5,6-pentafluorobenzyl)oxime derivatives which have chromatographic and ECD properties

TABLE VIII

Relative Retention Time of the Boronate Derivatives of Cortisone and Prednisone[a]

Steroid	3,5-*bis*(Trifluoromethyl)-benzeneboronate	Benzeneboronate	4-Bromobenzene-boronate
Cortisone	0.28	1.0[b]	2.35
Prednisone	0.29	1.0[c]	2.33

[a]Column, 3 ft. X 1/8" nickel, 1% OV-17 on Gas Chrom Q (100-120 mesh); temp, 280 C; flow rate, 60 ml min^{-1}, nitrogen.

[b]Retention time = 7.9 min.

[c]Retention time = 8.0 min.

superior to the pentafluorophenylhydrazone derivatives of steroids.

Alkyl and arylboronic acids have often been used to stabilize steroid diol groups toward GLC (26). Boronic acids with electron-capturing groups recently have been introduced and used for the trace level analysis of a variety of volatile bifunctional compounds (27-30). The derivatives of cortisone and prednisone formed with 3,5-*bis*(trifluoromethyl)-benzeneboronic acid, benzeneboronic acid, and 4-bromobenzeneboronic acid are formed readily at room temperature and are thermally stable at the high temperatures necessary for GLC (Table VIII). Notable is the high volatility of the 3,5-*bis*(trifluoromethyl)benzeneboronate derivatives, which are significantly more volatile than the benzeneboronates. Other related bifunctional steroids, hydrocortisone, aldosterone, 11-dehydrocorticosterone and 3β,17α,-20α-trihydroxy-5-pregn-ene either produce multiple peaks or give no products by GLC when allowed to react with the boronic acids. When silylation by either TMSI or BSTFA of any unprotected hydroxyl groups was attempted, there was evidence that partial cleavage of the boronate ester also occurred.

CONCLUSIONS

The electron-capture detector is an excellent detector for the trace level analysis of steroids. Some steroids are naturally electron-capturing and can be determined at low levels without modification provided they are thermally stable. For those steroids which are not electron-capturing, the technique of derivatization can be used to introduce an electrophore into the steroid structure, bringing them within the scope of the ECD.

ACKNOWLEDGMENT

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Selective Reactions in the Analysis and Characterization of Steroids by Gas Chromatography-Mass Spectrometry

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ABSTRACT

Gas chromatography-mass spectrometry (GC-MS) is a technique especially suitable for the analysis and characterization of steroids, and its power has been extensively demonstrated. The efficacy of GC-MS is limited, nevertheless, by the fact that steroid mixtures – whether of natural origin only, or augmented by synthetic analogs – often contain similar components that are poorly distinguished. The fortuitous overlap of gas chromatographic peaks from disparate compounds also impairs the definition of retention data. Controlled modification of the sample by means of selective reactions is therefore a valuable adjunct to the application of GC-MS. Two examples are discussed: (a) the enzyme cholesterol oxidase, isolated from various microorganisms, catalyzes the oxidation of many 3β-hydroxy-5-enes (with concomitant isomerization) to 4-en-3-ones; 3β-hydroxy-5α-steroids are also oxidized to the corresponding 3-ones, but other steroids (3α-hydroxy- or 5β-isomers, etc.) are unaffected. The mild conditions required (pH 7, 30 C) are advantageous for the analysis of sensitive steroids, and the retention index increments, as well as the mass spectra of the ketones, are characteristic. The enzyme accepts as substrates a wide range of 3β-hydroxysteroids, tolerating oxygenation in ring B and even catalyzing the oxidation of 2-oxacholesterol to the expected lactone; and (b) Steroids possessing 1,2-diol or 1,3-diol groupings include estriols, 2-hydroxyestrone, 20,22-dihydroxycholesterols, ecdysones, brassinolide and many corticosteroids. The selective formation of cyclic derivatives can provide several analytically useful features, such as convenient retention times, moderate mass increments (24 amu for a methaneboronate), distinctive mass spectra and usually abundant molecular ions. These are exemplified for 5-pregnene-3β,20,21-triols and for 20,22-dihydroxycholesterol as well as its enzymic oxidation product.

INTRODUCTION

Gas chromatography-mass spectrometry (GC-MS) has proved to be an especially apt technique for the analysis and characterization of steroids (1,2). One major reason for this lies in the substantial uniformity of the nuclear carbon skeleton, which leads to informative regularities in retention data for the various homologous and functionalized steroids encountered in nature or as synthetic products. The constitutional features typical of steroids – a relatively stable nucleus, side chains of distinctive types and different patterns of oxygenation and unsaturation – also, by their nature, afford useful mass spectrometric data (3,4). Separations and identifications of steroids by GC-MS can be further improved by exploiting the properties of suitable derivatives (1,2,5-8). Two useful monographs on derivatives of organic compounds for chromatography have been published (9,10).

It is frequently necessary to distinguish between steroids that differ only in the configuration of hydroxyl groups and/or of ring junctions. For the pure compounds, retention data may be definitive, but the complexity of natural steroid mixtures can render measurements quite uncertain. In such instances, a valuable adjunct to GC-MS is provided by the controlled modification of portions of the sample by means of selective reactions. Two examples are represented in this report: the application of enzyme-catalyzed oxidation to 3β-hydroxysteroids and the formation of cyclic boronate esters from steroidal α-glycols.

The enzyme cholesterol oxidase (11), derived from various bacterial or fungal species, catalyzes the oxidation of many 3β-hydroxy-5-enes (with concomitant isomerization) to 4-en-3-ones, and also of 3β-hydroxy-5α-steroids to the corresponding ketones (12,13). Isomers such as 3α-hydroxy or 5β-steroids remain unoxidized, as do hydroxyl groups elsewhere in the molecule, in general. The mild conditions of oxidation (pH 7, 30 C) facilitate studies of sensitive steroids, whereas the retention changes and the mass spectra of the products are highly characteristic of substrate type (14-17). These properties are of value, e.g., in the analysis of sponge sterols (18).

Many biologically active steroids and their metabolites contain 1,2-diol or 1,3-diol groupings in a variety of positions: e.g., estriol, 2-hydroxyesterone, 5β-pregnane-3α-17α,20α-triol, cortisol and 20,22-dihydroxycholesterols, whereas certain ecdysones (19) and the recently discovered plant hormone brassinolide (20) each contain one nuclear and one side-chain α-glycol group. For steroids of these classes, the selective formation of cyclic derivatives of the

diol components can provide several analytically powerful features. Special interest attaches to alkane- and arene-boronic acids, which readily react with 1,2-diols under mild, neutral conditions to form cyclic esters. As in the analogous formation of borates, the esterifications require very little activation energy and in many instances are readily reversible. Accordingly, quantitative analyses based on cyclic boronates are practicable only in favorable cases, but the derivatives are very suitable for qualitative studies. Cyclic methaneboronates (21) are particularly useful in affording convenient retention times, a modest mass increment of 24 amu/diol, and mass spectra that are usually characterized by well-defined molecular ions, as well as fragment ions exhibiting the distinctive ^{10}B:^{11}B isotope ratio (22-25).

EXPERIMENTAL PROCEDURES

Materials

Steroids were obtained from commercial sources except for the following: 24-methylenecholesterol (as propionate) from Dr. M. Barbier (Gif-sur-Yvette); 5-ergosten-3β-ol, 5α-stigmast-8(14)-en-3β-ol and 5-pregnene-3β,20,21-triols (20α and 20β) from the Medical Research Council Steroid Reference Collection; and 2-oxacholesterol from the G.D. Searle Co.

Methaneboronic acid was obtained from Alfa Inorganics Inc. and the trimethylsilylating reagents, N,O-*bis*(trimethylisilyl) trifluoroacetamide (BSTFA), N,O-*bis*(trimethylsilyl)acetamide (BSA), hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS), from Pierce Chemical Co. Ethyl acetate (EtOAc) was of Nanograde quality (Mallinckrodt), and isopropanol was of AnalaR grade (British Drug House). Acetic anhydride (AnalaR grade) was redistilled before use. Pyridine (AnalaR) was redistilled over potassium hydroxide pellets. N,N-Dimethylformamide (BDH AnalaR) was redistilled before use. Cholesterol oxidase (cholesterol O_2 oxidoreductase: EC 1.1.3.6), derived from *Nocardia erythropolis,* was obtained from the Boehringer Corporation Ltd. as a solution (1 mg/ml) in aqueous ammonium sulfate, representing 25 enzyme units/mg.

Gas chromatographic packings and stationary phases were obtained from Applied Science Laboratories and from Supelco Inc. Reactivials were from Pierce Chemical Co.

Methods: GLC and GC-MS

Gas liquid chromatography (GLC) was carried out with a Perkin-Elmer F-11 chromatograph (used for the boronate studies) and a Pye 104 instrument (used for cholesterol oxidase studies). Silanized glass columns (2 m x 4 mm id) were packed with 1% OV-1 or 1% OV-17 on Gas Chrom Q, 100-120 mesh; the flow rate of nitrogen carrier gas was 40 ml/min. Both instruments employed hydrogen flame ionization detectors. Open-tubular GLC was carried out with a Pye 104 chromatograph; the 25 m x 0.5 mm column was coated with OV-1 phase by the Silanox procedure (26); the flow rate of helium carrier gas was 2 ml/min; a falling-needle injection system was used (27).

Combined gas chromatography-mass spectrometry (GC-MS) was effected mainly on an LKB9000 instrument fitted with a silanized glass column (2 m x 4 mm id) of 1% OV-1 on Gas Chrom Q, 100-120 mesh; the helium carrier gas flow was 30 ml/min. Mass spectra were measured under electron-impact conditions: ionizing energy 20 eV (except that 2-oxacholesterol derivatives were run at 70 eV), accelerating voltage 3.5 kV and source temperature 260 C. Chemical ionization mass spectra were recorded on a Dupont 21-490F instrument as previously described (28).

Preparation of Derivatives

Trimethylsilyl ethers. For nonhindered hydroxyl groups, the steroid (100 μg) was treated either with BSA alone (for simple 3β-hydroxysteroids) or with a mixture of BSA/HMDS/TMCS (10:10:5 μl) and kept at 60 C for 30-60 min. Excess reagents were removed in a stream of nitrogen and the residue was taken up in EtOAc (100 μl). For hindered hydroxyl groups, the steroid was heated with BSA/HMDS/TMCS (60:60:10 μl) in a Reactivial at 140 C for 48 hr; the solution was diluted with EtOAc (30 μl) for GLC.

Boronate esters. Methaneboronic acid (1 mol proportion) in dry pyridine was added to the steroid diol (100 μg) and the mixture was kept at 60 C for 30 min. After removal of solvent under nitrogen, the residue was taken up in EtOAc (100 μl).

Trimethylsilylation in the presence of cyclic methaneboronate groupings. Method A: the steroid boronate, prepared as already described, was treated with BSTFA (5 μl) and heated at 60 C for 2 min. After evaporation to dryness, the residue was taken up in EtOAc (30 μl). Method B: the steroid boronate was heated with BSTFA in dimethylformamide (1:3 v/v, total vol 20 μl) at 60 C for 5 min; the solution was evaporated to dryness and the residue taken up in EtOAc (30 μl).

Acetates. The steroid (100 μg) was heated with acetic anhydride (15 μl) and pyridine at C for 30 min. After evaporation to dryness, the product was redissolved in EtOAc (100 μl).

Enzymic Oxidations

A solution (1 μg/μl) of the sterol (50-250 μg) in isopropanol was diluted with 50 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7.0) (3 ml). In some cases, Triton X-100 (1 mg/ml) was incorporated. After addition of cholesterol oxidase (2-10 μl), the mixture was incubated for 18 hr at 30 or 37 C. The products were extracted with EtOAc (4 x 1.5 ml), dried over anhydrous Na_2SO_4 and evaporated to dryness.

For 2-oxacholesterol, the rate of hydrogen peroxide production accompanying enzymic oxidation was monitored by the coupled oxidation method employing phenol, peroxidase and 4-aminoantipyrine, as previously described (15). The rate of oxidation was 15-20% of that for cholesterol under the same conditions. Preparative incubation was effected with 200 μg of 2-oxacholesterol and 20 μl of enzyme overnight. When the product was extracted with diethyl ether and examined by thin layer chromatography (TLC), it showed a single spot, less polar than the substrate. Isomerization to the α,β-unsaturated lactone was indicated by a strong ultraviolet absorption band at 225 nm in ethanol. The product was unaltered by trimethylsilylating reagents as indicated by the identity of the GLC behavior (I = 3235) before and after treatment.

RESULTS AND DISCUSSION

Selective Oxidations with Cholesterol Oxidase

Retention data associated with the application of cholesterol oxidase to a variety of sterols are given in Table I. Sterols **1-7**, comprising 3 Δ^5 and 4 5α-sterols, further illustrate the types of regularity that have been observed for a large number of previous examples (14-18). Retention index increments on the polymethylsiloxane phase OV-1 are ca. +120 for the conversion of 3β-hydroxy-5-enes to 4-en-3-ones, but only ca. +40 for the oxidation of 3β-hydroxy-5α-steroids. The corresponding increments from the trimethylsilyl ethers are ca. +45 (for Δ^5) and -40 (for 5α). These large differences are especially helpful in separating Δ^5 from corresponding 5α-sterols. As would be expected, however, the precise retention increments are not entirely unaffected by other structural features. We have previously noted that 5α-cholest-7-en-3β-ol and 5α-ergost-*E*22-en-3β-ol, unresolved on OV-1 as their trimethylsilyl ethers, are oxidized to readily separable ketones (ΔI = 15) (18). Similar small but significant variations in ΔI values are illustrated in Figure 1: the separation between the trimethylsilyl ethers of α-spinasterol and stigmastanol (ΔI = 12) is inferior to that for the corresponding ketones (ΔI = 27). Figure 1 also shows the convenience of the ketones as derivatives having retention times on OV-1 a little lower (or, with the 4-en-3-ones, a little higher) than those of the sterol trimethylsilyl ethers.

When additional hydroxyl functions are present in a 3β-hydroxysteroid, the unique selectivity of cholesterol oxidase is of even greater value. Successful oxidations at C-3 have

TABLE I

Retention Data for Sterols, Their Trimethylsilyl Ethers and Ketones Formed by Oxidation with Cholesterol Oxidase

		Retention data $I^{OV\text{-}1}_{265}$			ΔI	
	Sterol	(i)[a] 3-OH	(ii)[b] 3-OTMS	(iii)[b] Ketone	(i)→(iii)	(ii)→(iii)
1	5α-Cholest-7-en-3β-ol	3115	3195	3150	+35	-45
2	Brassicasterol	3115	3185	3235	+120	+50
3	24-Methylenecholesterol	3155	3230	3270	+115	+40
4	5-Ergosten-3β-ol	3165	3240	3290	+125	+50
5	α-Spinasterol	3245	3325	3280	+35	-45
6	5α-Stigmast-8(14)-en-3β-ol	3250	3320	3285	+35	-35
7	5α-Stigmastan-3β-ol	3260	3335	3300	+40	-35
8	6-Ketocholestanol	3340	3370	3345	+30	+5
9	22-Ketocholesterol	3255	3290	3380	+125	+90
10	2-Oxacholesterol	–	3070[c]	3235[c,d]	–	+165

[a]Packed column, 6 ft, 1% OV-1.

[b]Open tubular column, 25 m.

[c]Determined at 238 C.

[d]Lactone.

Systematic names: brassicasterol, 5,22-ergostadien-3β-ol; 24-methylene-cholesterol, 5,24(28)-ergostadien-3β-ol; α-spinasterol, 5α-stigmasta-7,E22-dien-3β-ol; 6-ketocholestanol, 3β-hydroxy-5α-cholestan-6-one; 22-ketocholesterol, 3β-hydroxy-5-cholesten-22-one; 2-oxacholesterol, 2-oxa-5-cholesten-3β-ol.

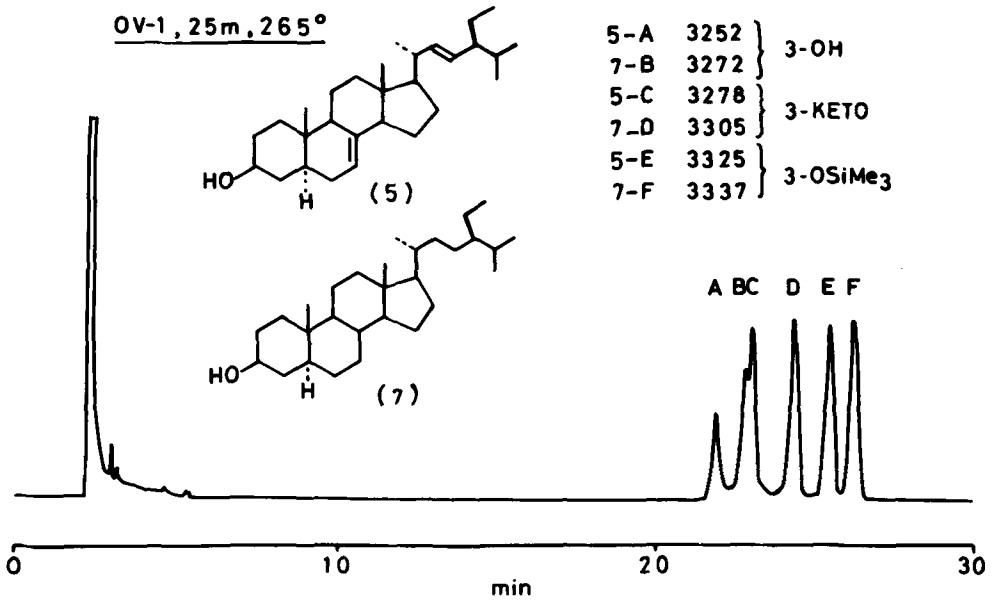


FIG. 1. GC separations of α-spinasterol and stigmastan-3β-ol, their corresponding ketones from *Nocardia erythropolis* enzyme oxidation and trimethylsilyl ethers on an open tubular column (25 m) coated with OV-1 at 265 C with a helium flow rate of 2 ml/min.

been demonstrated in the presence of as many as 3 other oxidizable hydroxyl groups (17), and also of the allylic 16-en-20α-ol (15) and 5-en-7α-ol (29) systems. This last example reflects another feature of the enzyme, i.e., its tolerance of certain substituents in ring B. A further illustration of this is provided by the ready conversion of 3β-hydroxy-5α-cholestan-6-one (Table I, no. **8**) into 5α-cholestane-3,6-dione. (The regularity of the retention increment is fortuitous, having regard to the expected vicinal effect of the 6-keto group).

Mass spectra of 3β-hydroxy-5α-cholestan-6-one (no. **8**) and of 22-ketocholesterol (no. **9**) are compared with those of their respective oxidation products in Figures 2 and 3. The main fragmentations of the 3β-hydroxy-6-ketone and the 3,6-dione (Fig. 2) are similar, with the exception of the ion at m/z 331 ($M-C_4H_7O$) which is prominent only in the 3-ol spectrum and which represents the loss of C-1/C-4 with one hydrogen transfer, as elucidated for 5α-cholestan-6-one by Djerassi et al. (30). In the case of 22-ketocholesterol, the oxidation product affords a more distinctive mass spectrum; the base peak of m/z 124 is well correlated with the 4-en-3-one group (31,32). The peak at m/z 99, indicative of the position of the keto group at C-22, is prominent in both spectra shown in Figure 3 while the nuclear stabilization conferred by the enone group enhances the abundance of the complementary ion (M-99).

As a consequence of the fact that cholesterol oxidase oxidations at C-3 proceed in the presence of other hydroxylic or ketonic groups, the enzyme facilitates the preparation of steroidal 4-en-3-ones, labeled elsewhere with isotopes. [4-^{14}C]-7α-Hydroxy-4-cholesten-3-one (29), [20-^{18}O]-20α/β-hydroxy-4-pregnen-3-one (33), and [16,16-$^{2}H_2$]-and [16,16,17-$^{2}H_3$]-testosterone (34) have thus been obtained via labeled 5-en-3β-ol precursors.

Finally, a striking demonstration of the extent of application of cholesterol oxidase is given by the successful oxidation of 2-oxacholesterol (10) to the lactone, 2-oxa-4-cholesten-3-one. Free 2-oxacholesterol (a hemiacetal) was decomposed by GLC, but the trimethylsilyl ether was stable and was examined by GC-MS. Under electron impact, the 3 major ions (Fig. 4) were at m/z 342 ($C_{25}H_{42}^{+}$; resulting from loss of $Me_3SiO{\cdot}CHO$ from ring A), m/z 370 (loss of Me_3SiOH) and m/z 229 (base peak: loss of side chain and of $Me_3SiO{\cdot}CHO$). The much simpler chemical ionization mass spectrum, measured with isobutane as reactant gas (Fig. 5), showed analogous fragmentations, ($MH-Me_3SiOH$) providing the base peak at m/z 371, with an increased abundance of the molecular ion in its protonated form at m/z 461. The mass spectra of the oxidation product, shown in Figure 6, reflect the greater stability expected of the molecular ion from a lactone

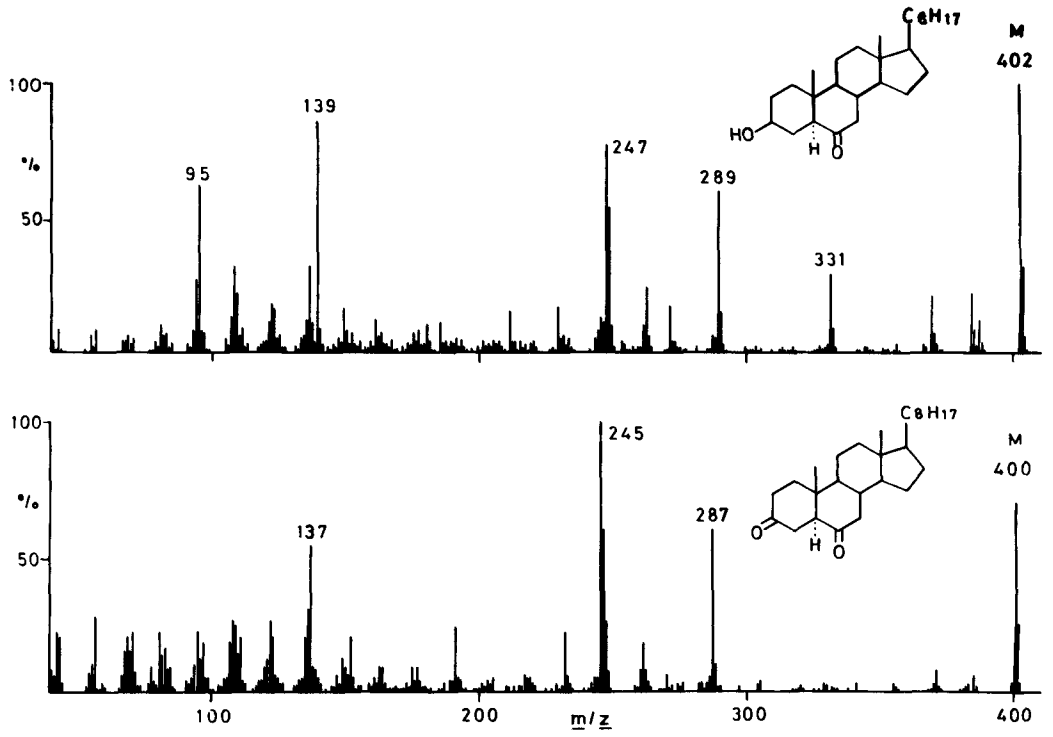


FIG. 2. Mass spectra (20 eV) or 3β-hydroxy-5α-cholestan-6-one (top) and 5α-cholestane-3,6-dione (bottom).

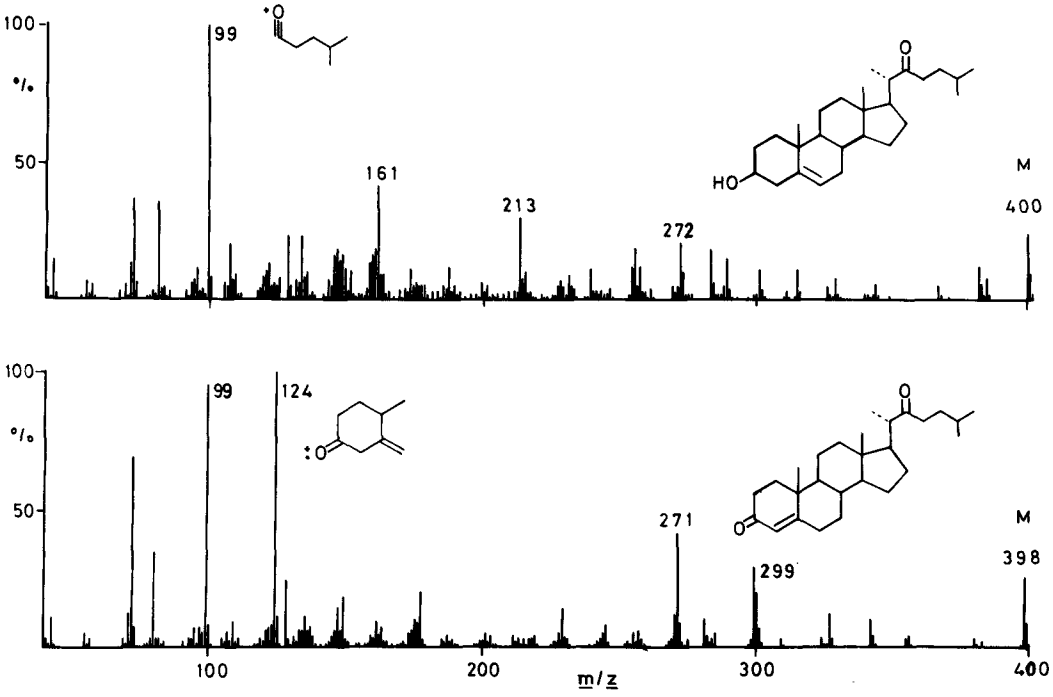


FIG. 3. Mass spectra (20 eV) of 22-ketocholesterol (top) and 22-keto-4-cholesten-3-one (bottom).

(m/z 386, 69% of base peak). Prominent ions in the electron impact spectrum, analogous to those of 4-en-3-ones, included m/z 342 (loss of CO_2, analogous to loss of ketene) and m/z 126 (Ion i), corresponding to m/z 124 (31,32), already mentioned. The identity of the base peak (m/z 93) has not yet been established. The isobutane chemical ionization spectrum of 2-oxacholestenone consisted essentially of the protonated molecular ion alone (m/z 387).

These examples give some indication of the versatility of cholesterol oxidase as a selective reagent for 3β-hydroxy steroids. It should be noted that the substrate selectivities of enzymes from different sources are not the same: in particular, the *Nocardia* enzyme has little effect on steroids of the androstane class, and its good substrates possess side chains of 2 or more carbon atoms. The enzyme from *Brevibacterium sterolicum* (35), on the other hand,

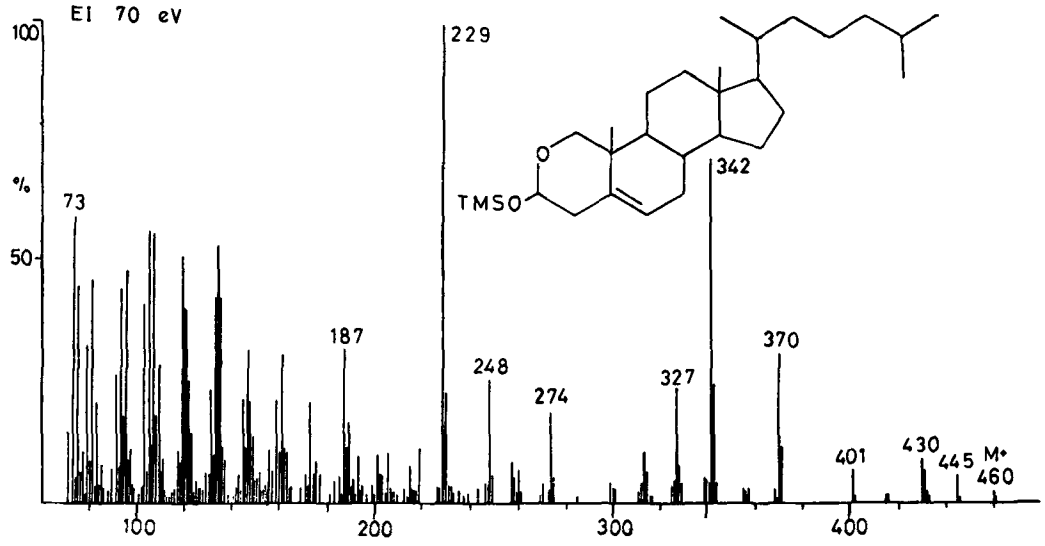


FIG. 4. Mass spectrum (70 eV) of 2-oxacholesterol trimethylsilyl ether.

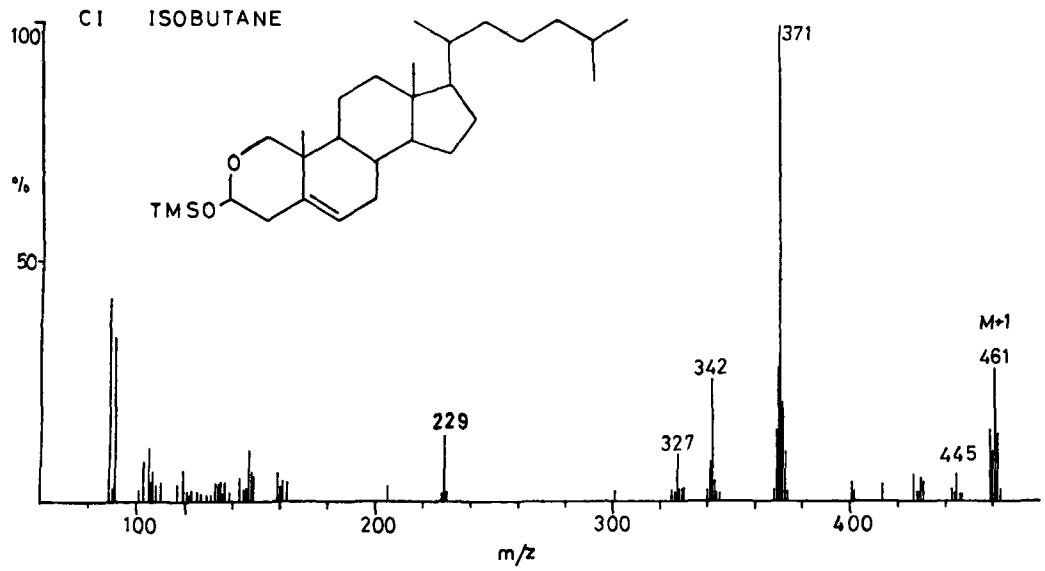


FIG. 5. Chemical ionization mass spectrum of 2-oxacholesterol trimethylsilyl ether, using isobutane as reactant gas.

catalyzes the oxidation of 5-androsten-3β-ols and 5α-androstan-3β-ols (17,36) and is thus applicable, e.g., to metabolites of androgenic steroids (17,36,37).

Selective Formation of Cyclic Boronates of Diols

The role of cyclic boronates in relationship to other derivatives is illustrated here with reference to a few typical steroids having α-diol groups in the side-chain.

Gas chromatographic data for various derivatives of 5-pregnene-3β,20α,21-triol (Structure **11**) and its 20β-epimer (Structure **12**) are given in Table II for OV-1 and OV-17 columns. The most striking feature is the marked reduction of retention time on OV-1 accompanying methaneboronate formation: the elution of this derivative some 250 retention index units lower than the tri-trimethylsilyl ether is illustrated in Figure 7. A closely similar decrement (ΔI = -245) was observed earlier for the derivatives of 20β,21-dihydroxy-4-pregnen-3-one (23). The 20,21-methaneboronate 3-trimethylsilyl ether, also included in the chromatogram in Figure 7, is obtainable by gentle trimethylsilylation, but

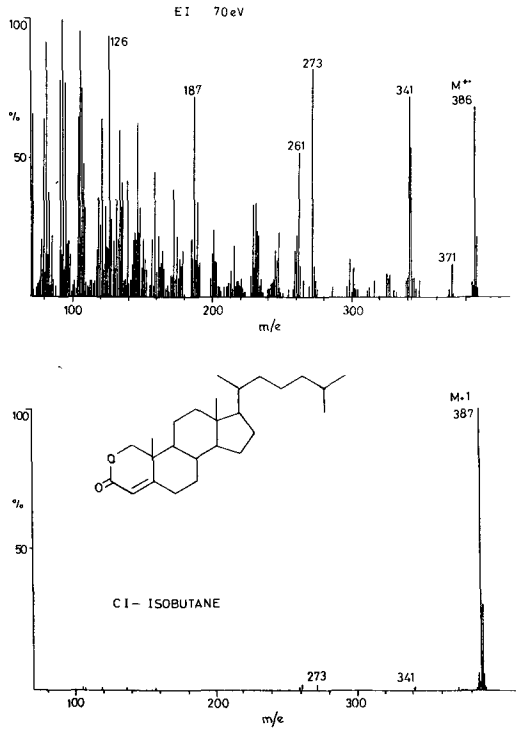
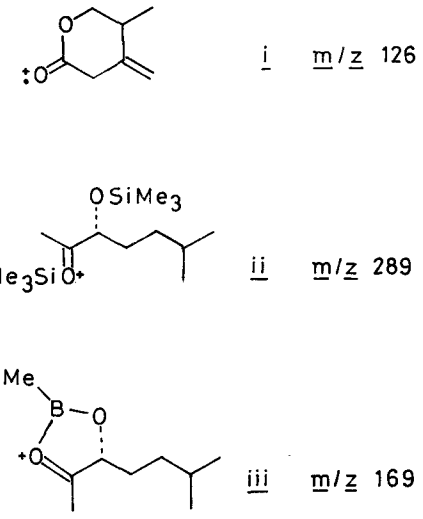


FIG. 6. Mass spectra of 2-oxa-4-cholesten-3-one recorded at 70 eV using electron impact (top) and isobutane chemical ionization (bottom).

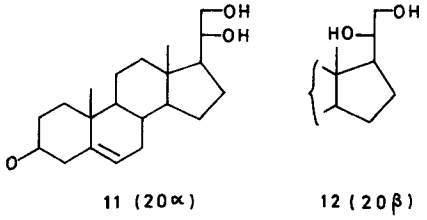


ION STRUCTURES

in the presence of excess reagent the boronate ring is ultimately replaced by trimethylsilyl groups. With regard to the separation of 20α/β epimers, most of the derivatives are effective.

Corresponding mass spectrometric results are shown in Table III. It is evident that the methaneboronates combine the advantage of low molecular weight with the property of yielding relatively abundant molecular ions. Molecular ions were undetectable for the acetates and trimethylsilyl ethers, but were prominent in the spectra of the free triols and methaneboronates.

In the mass spectra of the free triols, the most notable ions were at m/z 249 and 223, doubtless representing the (M-85) and (M-111) ions which result from cleavages of ring B and are typical of 5-en-3β-ols (38,39,40). Wyllie et al. (39) have observed these ions from 5-pregnene-3β,20β-diol. The data cited in Table III also disclose abundant ions at m/z 231 and 213, attributable to successive losses of H_2O from the fragment of m/z 249. In the spectra of the trimethylsilyl ethers, the base peaks resulted from elimination of all 3 silyl groups together with C-21, whereas other ions, particularly those at m/z 129 and (M-129) typifying the



11 (20α) 12 (20β)

TABLE II

Retention Indices[a] (I) and Increments (ΔI) for Derivatives of 5-Pregnene-3β,20,21-triols

	11 (20α-triol)		**12** (20β-triol)	
OV-1, 240 C	I	ΔI	I	ΔI
Triol	2960	–	2950	–
Triacetate	3230	+270	3225	+275
Tri-TMS ether	3090	+130	3110	+160
Me boronate	2835	- 125	2810	- 140
Me boronate/TMS	2895	- 65	2870	- 80
		OV-17,240 C		
Triol	3410	–	3400	–
Triacetate	3640	+230	3660	+260
TMS ether	3140	- 270	3165	- 235
Me boronate	3190	- 220	3155	- 245
Me boronate/TMS	3095	- 315	3080	- 320

[a]Values cited to the nearest 5 units.

5-en-3-ol parent steroid, were of low intensity. In contrast, the methaneboronate 3-trimethylsilyl ethers afforded a characteristic balance of fragmentation modes, yielding m/z 129 as base peaks, together with abundant ions at m/z 301 (M-129). The methaneboronates with free 3β-hydroxyls afforded base peaks by loss of H_2O from the molecular ion, in accordance with many earlier instances of steroid boronates having a free 3α- or 3β-hydroxyl group (22, 23,25).

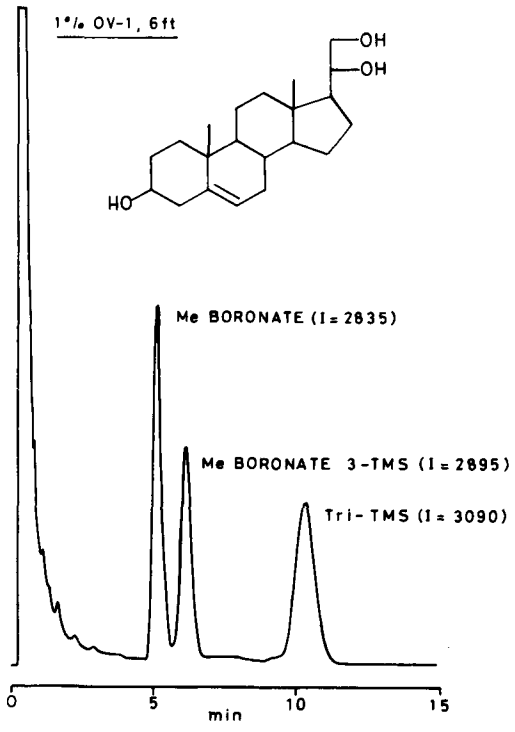


FIG. 7. GC separations of the methaneboronate, methaneboronate 3-trimethylsilyl ether and tri-trimethylsilyl ether derivatives of 5-pregnene-3β,20α,-21-triol on a packed column (2 m x 4 mm id) containing 1% OV-1 on Gas Chrom Q (100-120 mesh) at 240 C with a nitrogen flow rate of 40 ml/min.

One of the most important steroidal α-glycols is [20*R*,22*R*] 20,22-dihydroxycholesterol (Structure **13**), a major intermediate in the biogenesis of the steroid hormones from cholesterol. The tri-trimethylsilyl ether was effectively applied by Burstein et al. (41) for GC-MS to determine the mode of enzymic incorporation of oxygen at C-20 and C-22. Following incubation in the presence of $^{16}O_2$ and $^{18}O_2$, the ions of type ii produced by C-17/C-20 α-cleavage were monitored for relative abundance of the 3 possible isotopic forms. Figure 8 shows salient features of the 20 eV mass spectra, recorded by GC-MS, of the tri-trimethylsilyl ether ($I^{OV\text{-}1}_{265°}$ = 3490) and the 20,22-methaneboronate 3-trimethylsilyl ether ($I^{OV_5}_{265°}$ = 3295). Under these conditions, ions of type ii from the tri-trimethylsilyl ether amounted to ca. 4% of total ionization (above m/z 70). The preponderant cleavage was, as

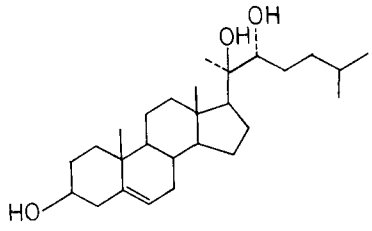


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TABLE III

Mass Spectral Data (20 eV) for 5-Pregnene-3β,20,21-triols and Derivatives*

	[M]+		Base peak[a]	Other principal ions: m/z (relative intensity in parentheses)																	
11	334	(70)	316[b]	298	(80)[c]	285	(60)[f]	283	(58)[e]	267	(50)[g]	255	(46)[o]	249	(45)[k]	241	(36)	231	(58)[m]	223	(36)[l]	213	(48)[n]
11A	460	(–)	400[b]	385	(5)[d]	340	(14)[c]	325	(7)[e]	280	(49)[h]	265	(32)	219	(16)	172	(58)	159	(55)	121	(53)	60	(45)
11B	550	(–)	267[g]	535	(0.1)	447	(4)	357	(5)[f]	281	(2)	225	(3)	211	(2)	157	(7)	147	(5)	133	(12)	129	(4)
11C	358	(22)	340[b]	343	(8)	325	(38)[d]	273	(26)[k]	265	(12)	255	(27)[o]	247	(24)[l]	232	(24)	219	(38)	159	(52)	121	(84)
11D	430	(8)	129	415	(1)	340	(20)[b]	325	(9)[d]	301	(28)[j]	255	(3)[o]	241	(7)	219	(4)	159	(5)	145	(6)	121	(9)
12	334	(64)	316[b]	298	(86)[c]	285	(50)[f]	283	(66)[e]	267	(54)[g]	255	(50)[o]	249	(46)[k]	241	(46)	231	(58)[m]	217	(40)	213	(56)[n]
12A	460	(–)	400[b]	385	(4)[d]	340	(11)[c]	325	(6)[e]	280	(37)[h]	265	(26)	219	(14)	172	(47)	159	(48)	121	(45)	60	(22)
12B	550	(–)	267[g]	535	(0.1)	447	(6)	357	(6)[f]	281	(2)	225	(2)	211	(2)	157	(5)	147	(4)	133	(8)	129	(3)
12C	358	(63)	340[b]	343	(27)	325	(55)[d]	273	(60)[k]	265	(13)	255	(20)[o]	247	(69)[l]	232	(17)	219	(26)	159	(40)	121	(48)
12D	430	(11)	129	415	(2)	340	(30)[b]	325	(13)[d]	301	(46)[j]	255	(4)[o]	241	(8)	219	(5)	159	(6)	145	(7)	121	(12)

*A=triacetates; B=tri-TMS ethers; C=methaneboronates; D=methaneboronate TMS ethers.

[a]Neglecting ions below m/z 40 (or 80 for TMS ethers).

[b]M–ROH [R = H, CH_3CO or $(CH_3)_3Si$].

[c]M–2ROH.

[d]M–(ROH + CH_3).

[e]–(2ROH + CH_3).

[f]M–ROH - CH_2OR.

[g]M–2ROH - CH_2OR.

[h]M–3ROH.

[j]M–$(CH_3)_3SiOCH{=}CH_2$.

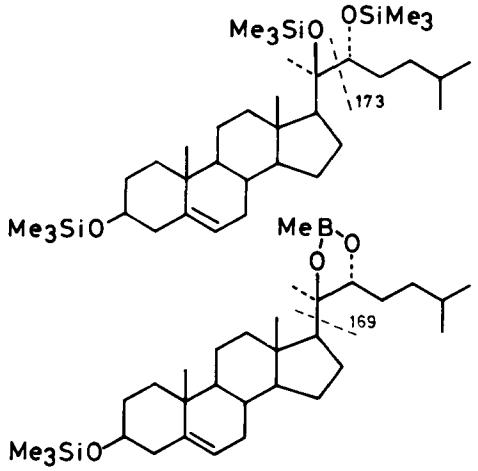
[k]M–85.

[l]M–111.

[m]M–103.

[n]M–121: ion $C_{16}H_{29}^+$.

[o]Nuclear ion $C_{19}H_{27}^+$.



m/z	%	Type
634	0.05	$M^{+\cdot}$
461	100*	$(M-173)^{+\cdot}$
289	10	side chain
514	15	$M^{+\cdot}$
169	100*	side chain

* each approx. 40% of Σ_{70}

FIG. 8. Characteristic mass spectral ions (20 eV) for the tri-trimethylsilyl ether and methaneboronate 3-trimethylsilyl ether derivatives of [20*R*,22*R*] 20,22-dihydroxycholesterol.

expected, between the 2 etherified positions (42). The methaneboronate trimethylsilyl ether, besides yielding a much more abundant molecular ion (lower in mass by 120 amu), afforded as base peak (40% of Σ_{70}) the boronate moiety (Ion iii) from C-17/C-20 side-chain cleavage.

The well established advantage of cyclic boronates for the enhancement of molecular ion abundance (21-25) has been further exploited by Lisboa et al. (43,44) in studies of [24*R*]-24,25-dihydroxycholecalciferol. (Such *seco*-steroids are thermally cyclized during GLC to the stable and separable *pyro*- and *isopyro*-isomers [45]). Both the 3-trimethylsilyl and the 3-*t*-butyl-dimethylsilyl ether of the 24,25-cyclic methaneboronate yielded abundant molecular ions in their 22.5 eV mass spectra. The fact that the boronate ring was adjacent to a secondary rather than a tertiary position no doubt accounts for the lack of any substantial α-cleavage to yield a boronate fragment analogous to Ion iii.

Finally, it is pertinent to mention the potential value of combining the use of cholesterol oxidase oxidation and of boronate ester formation. In a substrate such as 20,22-dihydroxycholesterol, addition of 1 mol proportion of methaneboronic acid affords a cyclic ester stable towards GLC ($I^{OV\text{-}1}_{265^\circ}$ = 3245), but if excess reagent is added, esterification at the isolated hydroxyl group can interfere with satisfactory GLC (21). The problem usually can be overcome by trimethylsilylation or acylation of the isolated hydroxyl group, which under mild conditions displaces the acyclic boronate group but not the cyclic group. In the case of 3β-hydroxysteroids with α-diol systems elsewhere in the molecule, prior selective oxidation to the 4-en-3-one is an alternative procedure, e.g., 20,22-dihydroxycholestenone (Structure **14**, Fig. 9) was readily formed by enzymic oxidation of **13**, and the 20,22-methaneboronate (Structure **15**) ($I^{OV\text{-}1}_{265^\circ}$ = 3380) yielded Ion iii (m/z 169: Fig. 9) as base peak. The quasi-complementary nuclear fragment at m/z 272 (M-169 + H) and the characteristic 4-en-3-one ion at m/z 124 were also abundant.

It has been possible, within the compass of this report, only to sketch a few of the modes of application of selective reactions in the analytical characterization of steroids. In conclusion, it may be pertinent to note that sample transformations by selective reagents are generally by far the simplest and most economical means of amplifying the evidence obtainable by GC-MS.

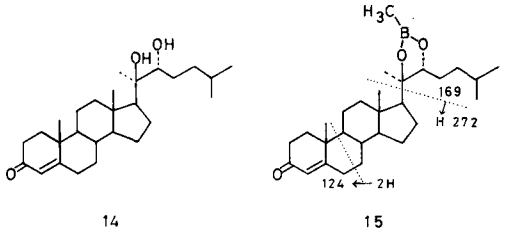


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FIG. 9. Structures of [20*R*,22*R*] 20,22-dihydroxy-4-cholesten-3-one and its corresponding methaneboronate illustrating side-chain and nuclear fragmentation processes.

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Identification of Steroids by Chemical Ionization Mass Spectrometry

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ABSTRACT

Chemical ionization (CI) mass spectra of various natural and synthetic steroids have been studied using methane, isobutane, ammonia, trideuterioammonia and hydroxy anion as reagent gases. The CI spectra of steroids give simple and well characterized ions, which provide information about molecular weight as well as functionalities in the molecules. Trideuterioammonia exchanges rapidly with active hydrogens (e.g., OH, SH, COOH, NH_2) in steroid molecules in the CI reaction and thus provides a convenient means of active hydrogen determination by mass spectrometry. Application of various CI processes to the analysis of steroids and conjugates have been made. Low levels of hydroxycholesterols in biological samples and in cholesterol autoxidation products were identified by the 4 ion patterns, $[M+NH_4]^+$, $[M-OH+NH_3]^+$, $[M-OH]^+$ and $[M-H_2O-OH]^+$, in ammonia CI. The position of hydroxy functions in the cholesterol side chain can be identified from the methane CI of hydroxycholesterol trimethylsilyl (TMS) derivatives. Sterol carboxylic esters can be identified as the ammonium adduct ion of the intact molecule, $[M+NH_4]^+$, in ammonia CI. Isobutane and hydroxy anion CI spectra of the steroid esters give abundant ion fragments of both steroids and carboxylic acid moieties. Identification of free bile acids and steroid glycosides without derivatization is also feasible with the CI process when ammonia is used as reagent gas.

INTRODUCTION

Mass spectrometry (MS) qualifies especially for analytical applications because of its very high sensitivity and because of the wealth of information afforded, quite unattainable by any other routine method of instrumental analysis for minute sample size. Its application to the analysis of traces is almost indispensable, especially in environmental, biological and medical sciences.

Identification, proof of structure and elucidation of fragmentation processes of steroids by electron impact (EI) MS is well established (1-3). In contrast, MS conducted by the relatively more gentle process of chemical ionization (CI) is much less described. The CI-MS offers several advantages which provide structural information unattainable by the EI process. In conventional ionization by EI, limitations or difficulties may arise because the sample yields molecular ions, M^+, insufficiently stable to permit detection, or because insufficiently volatile or thermally labile compounds may pyrolyze or isomerize before ionization. The EI process often results in complex ion spectra which, on one hand, may provide abundant information for molecular structure but, on the other hand, may result in an equivocal interpretation, especially in the presence of ions from impurities.

In CI, a reagent gas, e.g., methane, isobutane, or ammonia, is first ionized by electron impact. The ions formed in ion-molecule reactions, e.g., CH_5^+, $C_2H_5^+$ for methane, $C_4H_9^+$ for isobutane, and NH_4^+ for ammonia, then react chemically with the substrate, M, in fast acid/base-type reactions, and/or subsequently fragment to various extents. In comparison to the EI, the spectra obtained by the relatively more gentle CI process are simple and contain ions of molecular entirety. Therefore, the CI spectra are easy to interpret and the substrate molecules are often easily characterized from intense quasi-molecular ions such as the protonated ion $[M+H]^+$, hydride abstraction ion $[M-H]^+$, or adduct ion $M+NH_4^+$. In addition, the amount of CI fragmentation can be controlled within a wide range, where the proportion of information about molecular weight on the one hand and structure on the other hand can be adjusted to requirements, almost at will by varying the experimental conditions, such as changing reagent gases or ion source temperatures. Thus, the CI process often provides a versatile alternative to EI ionization (4-6).

The possibility of making the ion-molecule reaction between reagent gas and individual functional group of a compound selective is a promising aspect of CI operation. We have previously reported that selective ion-molecule reactions between the reagent gas and individual functional groups of various biochemical molecules can be achieved, and functional groups in the molecule can be recognized from the CI spectra (7). The method was further extended to an effective determination of active hydrogen in biochemical molecules by using trideuterioammonia as reagent gas (8). An investigation of the hydroxyl ion negative CI

mass spectra of C_{27} steroids having various functionalities has indicated that the sensitivity could be enhanced for some steroids (9). In this report, I will discuss the application of various CI processes to the identification of steroids and their conjugates.

EXPERIMENTAL

Materials

Steroids examined were either commercial samples from Steraloids Inc., Wilton, NH, or Research Plus Laboratories Inc., Denville, NJ, or they were prepared in our laboratories. All samples were analyzed by thin layer chromatography (TLC) and gas chromatography (GC) before use, and in those cases where purity was in doubt the samples were purified by high pressure liquid chromatography (HPLC) using Waters Associates Inc. (Milford, MA) equipment with 2 μ-Porasil columns (4 mm x 30 cm) connected in series and hexane-2-propanol (24:1) as the mobile phase.

Perdeuterated ammonia (99% deuterium) was obtained from Merck, Sharp and Dohme Canada Ltd., Montreal.

Mass Spectrometry

Mass spectra were obtained with a Finnigan Corp. Model 3200 quadrupole mass spectrometer equipped with EI and CI capabilities. Samples (0.2-1.0 μg) in a 1-cm capillary were introduced by direct, solid probe into the ionization chamber, with gradual heating from ambient temperature to 200 C to vaporize the sample. Ammonia and trideuterioammonia CI mass spectra were obtained using an ion-source temperature of ca. 100 C (uncorrected meter reading 70 C) and gas pressures between 0.3 and 0.5 Torr, adjusted to maximize the intensity of the $[NH_4]^+$ (m/e 18) ion. Higher pressures (up to0.9 Torr) gave diminished intensity of the $[NH_4]^+$ ion but increased intensities of the $[N_2H_7]^+$ (m/e 35) and $[N_3H_{10}]^+$ (m/e 52) ions. Some spectra were also recorded with an ion source of 195 C (uncorrected reading 150 C). Methane and isobutane CI mass spectra were obtained with an ion-source temperature of ca. 100 C and gas pressure of 0.4 Torr, adjusted to maximize the intensities of the $[CH_5]^+$ (m/e 17) and $[C_4H_9]^+$ (m/e 57) ions, respectively.

RESULTS

Recognition of Functional Groups by CI Mass Spectra

Spectra derived from the use of methane or isobutane as reagent gas, commonly used in CI-MS, contain 3 major ion types: the protonated molecular ion $[M+H]^+$, the hydride abstraction ion $[M-H]^+$ and the elimination ion $[M-X]^+$ (Table I).

Steroid olefins Ib and Ic gave both $[M+H]^+$ and $[M-H]^+$ ions, but the saturated hydrocarbon Ia gave only $[M-H]^+$ ions. Steroid ketones gave the $[M+H]^+$ ion predominantly. The ratio of the ion abundances $[M+H]^+/[M-H]^+$ is dependent on the proton affinity of a given functional group and on the relative stability of the ion species. Thus, the higher proton affinity of the diene (Ic) compared to the olefin Ib is reflected in the much higher $[M+H]^+/[M-H]^+$ ratio of Ic. Monofunctional alcohols gave 3 ions: $[M+H]^+$, $[M-H]^+$, and $[M-OH]^+$ as a general matter. The elimination ion $[M-OH]^+$ was the principal ion, except for Ii which gave the most abundant $[M-H]^+$. The exceptionally high abundance of the abstraction ion $[M-H]^+$ for Ii must result from the formation of a thermodynamically stable protonated ion of an α,β-unsaturated ketone; the $[M-H]^+$ is equivalent to the stable $[M+H]^+$ of Ie. The bifunctional diol Ij gave an additional double elimination ion $[M-H_2O-OH]^+$, indicating the presence of 2 hydroxyl functions. Cholesteryl esters, Ik and Il, gave neither $[M+H]^+$ nor $[M-H]^+$ ions, presumably because of facile elimination of the homoallylic hydroxyl ester function, but gave elimination $[M-X]^+$ as principal ions.

Ammonia CI spectra of steroids are most instructive, generally yielding abundant ions of 4 types: an ammonium adduct $[M+NH_4]^+$, an ammonia displacement ion $[M-X+NH_3]^+$, a protonated molecular ion $[M+H]^+$ and an elimination ion $[M-X]^+$ (Table I). Ammonia gas, with its high proton affinity (207 Kcal/mol [10]) and low level of energy transfer in ion-molecule reactions, forms abundant ammonium adducts $[M+NH_4]^+$ with many electronegative functional groups or it forms protonated molecular ions $[M+H]^+$, which are instructive for molecular weight determination and characterization of functional groups. Steroid olefins Ib and Ic display ions $[M+NH_4]^+$ and $[M+H]^+$, separated by 17 amu; the monoolefin Ib, having $[M+NH_4]^+$, and the conjugated diene Ic, having $[M+H]^+$ as principal ions. Likewise, steroidal ketone Id-If have these same 2 ions. The conjugated ketones Ie and If gave abundant $[M+H]^+$ ions, whereas the saturated ketone Id gave the ammonium adduct $[M+NH_4]^+$ as principal ion. Distinguishing 4-cholest-ene-3-one from its unconjugated isomer 5-cholest-ene-3-one by a similar CI technique was also reported previously (11). Steroidal alcohols Ih and Ii are characterized by 3 prominent high-mass ions, the highest being

TABLE I

CI (Methane, Isobutane and Ammonia) Mass Spectra of Steroids

Steroids		Methane and isobutane, m/e (%)				Ammonia, m/e (%)				
		$[M+H]^+$	$[M-H]^+$	$[M-X]^+$	Others	$[M+NH_4]^+$	$[M+H]^+$	$[M-X+NH_3]^+$	$[M-X]^+$	Others
Ia 5α-Cholestane	CH_4	–	371(100)	–	357(28)[a]					
Ib 5-Cholest-ene	CH_4	371(35)	369(100)	–	355(35)[a]	388(100)	371(25)	–	–	–
Ic 3,5-Cholesta-diene	CH_4	369(95)	367(100)	–	353(36)[a]	386(30)	369(100)	–	–	–
	iC_4H_{10}	369(100)	367(35)	–	–					
Id 5α-Cholestan-3-one	CH_4	387(100)	385(25)	–	–	404(100)	387(5)	–	–	–
Ie 4-Cholest-en-3-one	CH_4	398(100)	383(25)	–	–	402(100)	385(78)	–	–	–
If 4,6-Cholesta-dien-3-one	CH_4	385(100)	381(10)	–	–	400(5)	383(100)	–	–	–
	iC_4H_{10}	383(100)	–	–	–					
Ig 5α-Cholestan-3β-ol	CH_4	389(5)	398(25)	371(100)	–	406(100)	–	388(36)	–	–
Ih 5-Cholest-en-3β-ol	CH_4	387(10)	385(60)	369(100)	367(10)[b]	404(100)	–	386(95)	369(95)	–
	iC_4H_{10}	387(5)	385)10)	369(100)	–					
Ii 4-Cholest-en-3β-ol	CH_4	–	385(100)	369(50)	367(20)[b]	404(10)	–	386(28)	369(100)	385(21)[c]
Ij 5-Cholest-en-3β,7α-diol	CH_4	403(5)	401(10)	385(100) 367(20)[d]	–	420(3)	–	402(20)	385(25) 367(100)[d]	–
Ik Cholesterol 3β-acetate	CH_4	–	–	369(100)	367(40)[b] 353(15)[e]	446(100)	–	386(5)	369(10)	–
Il Cholesterol 3β-oleate	iC_4H_{10}	–	–	369(100)	283(30)[f]	668(100)	–	386(5)	369(25)	–

[a] M-CH_3.
[b] M-HX-H.
[c] M-H.
[d] M-HX-X.
[e] M-HX-CH_3.
[f] $C_{17}H_{33}COOH$+H.

the even-mass ammonium adduct $[M+NH_4]^+$, separated from the second even-mass $[M-OH+NH_3]^+$ by 18 amu, separated in turn from the lowest odd-mass ion $[M-OH]^+$ by 17 amu. The spectra of the saturated alcohol Ig at a temperature of 100 C, as shown in Table I, gave only 2 ions, $[M+NH_4]^+$ and $[M-OH+NH_3]^+$; however, with a higher source temperature of 195 C, the third ion, the elimination ion $[M-OH]^+$, was observed. With diol Ij, double elimination occurred and a fourth ion $[M-H_2O-OH]^+$ appeared in the spectrum.

Cholesteryl carboxylic acid esters Ik and Il, for which molecular or protonated molecular ions are absent in EI or methane and isobutane CI processes, are clearly characterized by the 3 diagnostic ions $[M+NH_4]^+$, $[M-X+NH_3]^+$ and $[M-X]^+$. Recognition of functionality in non-steroid compounds by these means also appears to be possible (7).

Determination of Active Hydrogen by CI-MS

Active hydrogen determinations by CI-MS with deuterium-labeled reagent gases have been recorded (12-14). We have studied CI spectra of active hydrogen-containing molecules in more detail using ammonia and trideuterioammonia (ND_3) as reagent gases (8). Ammonia CI mass spectra of steroids, as mentioned in the preceding sections, are dominated by 4 types of ions: $[M+NH_4]^+$, $[N+H]^+$, $[M-X+NH_3]^+$, and $[M-X]^+$. Analogous ions were found when ND_3 was used as reagent gas, but in analytes containing active hydrogen there was a rapid exchange of hydrogen for deuterium of the isotopic reagent gas ND_3 as well. Comparison of ions in NH_3 and ND_3 CI spectra yields a direct measure of the number of active hydrogens in a molecule. A typical example of an active hydrogen determination is given by the NH_3 and ND_3 CI of cortisol, as shown in Figure 1.

Comparison of the ammonium adduct m/e 380 and the protonated molecular ion m/e 363 in the NH_3 CI spectrum with that of m/e 387 and m/e 367 in the ND_3 CI spectrum quickly reveals the presence of 3 active hydrogens in cortisol. The ions m/e 320 and 303 in the NH_3 CI spectrum resulting from cleavage at the C_{17}-C_{20} bond of cortisol had been shifted to m/e 325 and 305, respectively, which further confirms the ion species of the fragment ions. Thus, the ND_3 CI spectra appear to provide information for the assignment of ion fragmentation processes as well.

All active hydrogens bonded to heteroatoms in aliphatic alcohols, phenols, carboxylic acids, amines, amides and mercaptans undergo complete exchange with deuterium in NH_3 CI spectra (8).

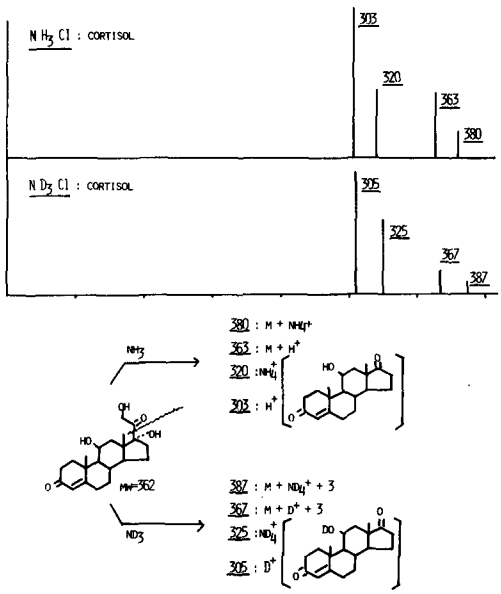

FIG. 1. NH_3 and ND_3 CI spectra of coritsol.

Identification of Hydroxycholesterols

Hydroxylations are important steps in cholesterol metabolism. In connection with our interests in the biological significance of hydroxycholesterols, it is important to develop an efficient technique to detect and identify them at low levels in complex biological samples. In this connection, we have developed methods for the separation and identification of hydroxycholesterols that combine HPLC and MS.

Hydroxycholesterols can be conveniently identified by CI-MS using methane, isobutane or ammonia as the reagent gas. Methane or isobutane CI spectra show 4 characteristic ions: the protonated molecular ion $[M+H]^+$, the hydride abstraction ion $[M-H]^+$, the single elimination ion $[M-OH]^+$ and the double elimination ion $[M-H_2O-OH]^+$. However, little information with regard to the position or nature of hydroxy function can be deduced from the methane or isobutane CI spectra.

Ammonia CI spectra of hydroxycholesterols, which are shown in Table II, are characterized by 4 prominent high-mass ions (at m/e 420,402, 385 and 367), the highest being the even-mass ammonium adduct $[M+NH_4]^+$, separated from the second even-mass $[M-OH+NH_3]^+$ by 18 amu, which is separated in turn from 2 odd-mass ions $[M-OH]^+$ and $[M-H_2O-OH]^+$ by 17 amu and 18 amu, respectively. These 4 ions characterize the molecular weight as well as the presence of 2 hydroxyl functions in the hydroxycholesterols.

TABLE II

NH_3 CI of Hydroxycholesterols

	m/e (%)			
	$[M+NH_4]^+$	$[M-OH+NH_3]^+$	$[M-OH]^+$	$[M-H_2O-OH]^+$
1α-Hydroxy-5-cholest-en -3β-ol	420(100)	402(7)	385(5)	367(2)
1β	420(200)	402(7)	385(12)	367(4)
4β	420(100)	402(43)	385(3)	367(4)
7α	420(3)	402(20)	385(25)	367(100)
7β	420(2)	402(5)	785(9)	367(100)
12α	420(100)	402(27)	385(33)	367(10)
16	420(100)	402(11)	385(18)	367(4)
17α	420(9)	402(66)	385(100)	367(18)
19	420(100)	402(11)	385(12)	367(3)
20α	420(5)	402(93)	385(100)	367(16)
21	420(100)	402(15)	385(19)	367(3)
22	420(100)	402(20)	385(23)	367(4)
23	420(100)	402(32)	385(37)	367(3)
24	420(100)	402(21)	385(17)	367(5)
25	420(100)	402(36)	385(27)	367(7)
26	420(100)	402(83)	385(75)	367(11)
5α-Hydrox-cholest-6-en -3β-ol	420(1)	402(4)	385(76)	367(100)

The relative abundance of the 4 ions in NH_3 CI spectra provides some information with regard to the nature of the hydroxyl functions in the molecules. The presence of an allylic hydroxyl function, as in 7α- and 7β-hydroxy-5-cholest-ene-3β-ol and 5α-hydroxy-6-cholest-ene-3β-ol, results in the production of abundant double-elimination ions $[M-H_2O-OH]^+$ at the m/e 367. The presence of a nonallylic tertiary hydroxyl function, as in 17α- and 20α-hydroxycholesterol, results in the production of an abundant single-elimination ion $[M-OH]^+$ at m/e 386. However, little information with regard to the position of hydroxy functions in the other hydroxycholesterols could be provided from the CI spectra.

The effect of the position of the hydroxy group on the fragmentation pathway of steroid alcohols under EI conditions has been thoroughly studied on the model of hydroxy derivatives of progesterone (15,16). The EI spectra of hydroxycholesterols studied in our laboratory show that the fragmentation ions of most hydroxycholesterols provide either weak or insignificant ions, which could be used to characterize the position of hydroxy functions.

TMS ethers have been extensively used for the characterization of sterols by GC-MS. The identification of the position of hydroxy functions of some sterols by EI spectra of the sterol TMS derivatives was possible because favorable cleavages occurred at the α-carbon of the TMS ether functions (17-19).

The methane CI spectra of hydroxycholesterol TMS ethers, shown in Figure 2, are much simpler than the reported EI-MS of some sterol TMS derivatives (17-19), where extensive fragmentations were recorded. Each hydroxycholesterol shown in Figure 2 exhibits a characteristic base peak resulting from a simple

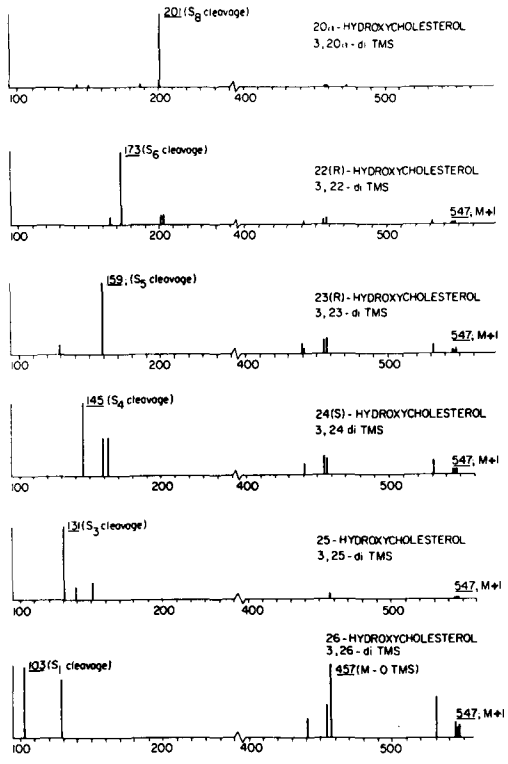

FIG. 2. CH_4 CI spectra of hydroxycholesterol TMS derivatives.

α-cleavage of the TMS ether groups. Thus, spectra of 20α-hydroxycholesterol gave the principal ion m/e 201 ($[CH_3]_3$ $SiOC[CH_3]$ $CH_2CH_2CH_2CH[CH_3]_2^+$, C_8-cleavage) resulting from the cleavage at C_{17}-C_{20} bond; 22-hydroxylcholesterol gave m/e 173 (C_6-cleavage); 23-hydroxycholesterol m/3 159 (C_5-cleavage); 24-hydroxycholesterol m/e 145 (S_4-cleavage); 25-hydroxycholesterol m/e 131 (S_3-cleavage; and 26-hydroxycholesterol gave m/e 103 (S_1-cleavage). Thus, the position of hydroxy functions in the side chain of cholesterol can be identified unambiguously.

Identification of hydroxysterols by the CI techniques already discussed are being used frequently in our laboratory for the detection of low levels of hydroxycholesterols in complex plasma lipids and other biological samples or in complex mixtures of cholesterol autoxidation products.

CI Mass Spectra of Sterol Carboxylic Acid Esters

Sterol carboxylic acid esters are generally identified, after hydrolysis, by their individual free sterols and carboxylic acids. With the improvement in HPLC separation and successful ionization by CI-MS, it is feasible to identify intact sterol carboxylic acid esters. MS of cholesteryl oleate in both EI and CI modes are shown in Figure 3.

The EI spectra of cholesteryl oleate show the dehydrated cholesterol peak m/e 368 ($M-C_{17}H_{33}COOH$) as the highest detectable mass ion, accompanied by abundant fragmentation ions in the low mass region. In contrast, the mild CI process using isobutane as reagent gas shows only 2 ions: m/e 369, the elimination ion $[M-C_{17}H_{33}COO]^+$, and m/e 283, the protonated carboxylic acid ion $[C_{17}H_{33}COOH+H]^+$. No mass ion of unfragmented cholesteryl oleate could be observed even at the mild CI process with isobutane. However, the 2 simple and unique ions in isobutane CI are characteristic for both cholesterol and oleic acid in the molecule of cholesteryl oleate. Since the carboxylic acid moiety of cholesteryl ester can be conveniently identified by a single protonated mass ion in isobutane CI, different carboxylic acid moieties in a mixture of cholesteryl esters can be identified simultaneously. Such an approach has been used successfully by Murata et al. (20) in the identification of cholesteryl esters in blood serum and egg yolk.

When the mildest chemical ionization gas, ammonia, was used, the mass spectrum of cholesteryl oleate gave the molecular ammonium adduct ion $[M+NH_4]^+$, m/e 668,

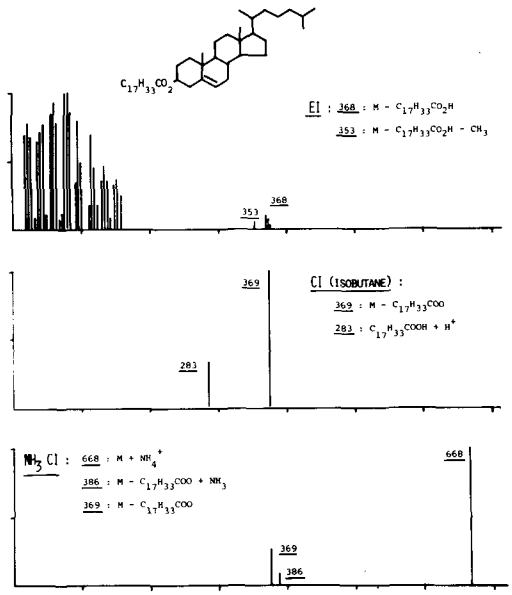

FIG. 3. EI (70 eV) and CI (i-C_4H_{10} and NH_3) spectra of cholesteryl oleate.

as the principal ion. Thus, the molecular weight of the ester can be characterized. Two additional ions, m/e 386 and 369, are the substitution ion $[M-C_{17}H_{33}COO+NH_3]^+$ and the elimination ion $[M-C_{17}H_{33}COO]^+$, respectively.

In contrast to isobutane CI, ions resulting from the carboxylic acid moieties of steroid esters in ammonia CI spectra were either weak or absent. Both isobutane and ammonia CI spectra are being used to complement each other for characterization of various sterol carboxylic acid esters (Lin et al., unpublished results).

Identification of sterol carboxylic esters may be carried out more favorably by negative chemical ion (NCI) MS. The OH^-NCI spectrum of cholesteryl oleate gave m/e 649 (100%) $[M-H]^-$, m/e 281 (45%), $[C_{17}H_{33}COO]^-$, m/e 263 (6%) and m/e 367 (8%). The sensitivity of cholesteryl esters in OH^- NCI spectra is ca. 7 times higher than that in CH_4 CI spectra (9). The more sensitive nature of NCI-MS of steroid esters may be attributed to the fact that carboxylic acid and ester functions are more susceptible to the formation of stable negative ions than of positive ions.

CI-MS of Bile Acids

Analysis of bile acids by CI with methane or isobutane as reagent gas has been carried out with derivatized bile acids methyl esters and methyl ester acetates (21,22). However, identi-

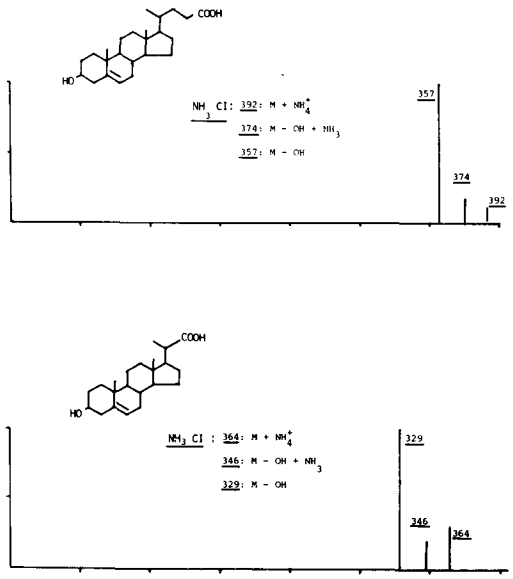

FIG. 4. NH_3 CI spectra of 3β-hydroxy-5-cholenic acid and 3β-hydroxybisnor-5-cholenic acid.

fication of bile acids without derivatization should be feasible using a combination of HPLC separation and CI-MS. Ammonia CI of 3β-hydroxy-5-cholenic acid and 3β-hydroxybisnor-5-cholenic acid are shown in Figure 4.

Three types of ions were observed in the ammonia CI spectra: the molecular ammonium adduct ion $[M+NH_4]^+$, the substitution ion of hydroxy function $[M\text{-}OH+NH_3]^+$ and the elimination ion of hydroxy function $[M\text{-}OH]^+$. The 3β-hydroxybisnor-5-cholenic acid (Fig. 4) has been found among the autoxidation products of cholesterol by HPLC separation and subsequent identification by the NH_3 CI (Ansari et al., unpublished results).

CI-MS of Steroid Glycosides

Steroid glycosides are quite nonvolatile and therefore derivatization is almost mandatory for successful mass spectrometric studies, except when relying on field desorption (FD) or plasma desorption-absorption techniques. In conventional EI-MS, ion fragmentation, even with derivatized steroid glycosides, is so extensive (23,24) that, e.g., the EI spectra of estradiol-3-glucoronide permethyl ether provide a very small molecular ion (25). We have recorded the CI spectra of some estradiol and estriol glycosides permethyl ethers using methane and ammonia as reagent gases. The results are given in Figures 5 and 6.

The methane CI spectra of the steroid glycosides (Fig. 5) show fairly abundant quasi-molecular ions $[M+H]^+$ or $[M\text{-}H]^+$. It should be noted that discrimination between the aromatic alcohol glycosides (the 3-glycosides) and the aliphatic alcohol glycosides (the 16- or 17-glycosides) is quickly accomplished by methane CI spectra. The 3-glycosides give only the hydride abstraction molecular ion $[M\text{-}H]^+$, whereas the 16- or 17-glycosides give only the protonated molecular ion $[M+H]^+$. The ammonia CI spectra of steroid glycosides (Fig. 6) gave most abundant molecular ammonium adducts $[M+NH_4]^+$ as the result of a mild ionization process.

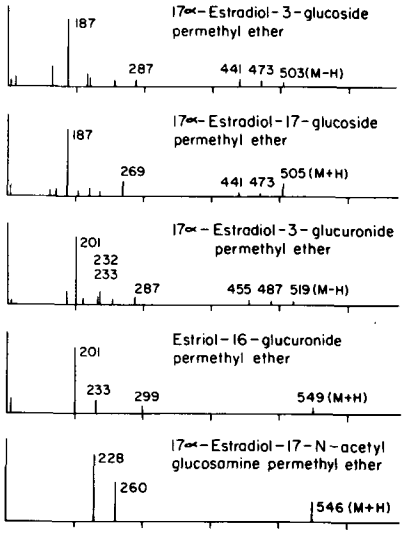

FIG. 5. CH_4 CI of steroid glycoside permethyl ethers.

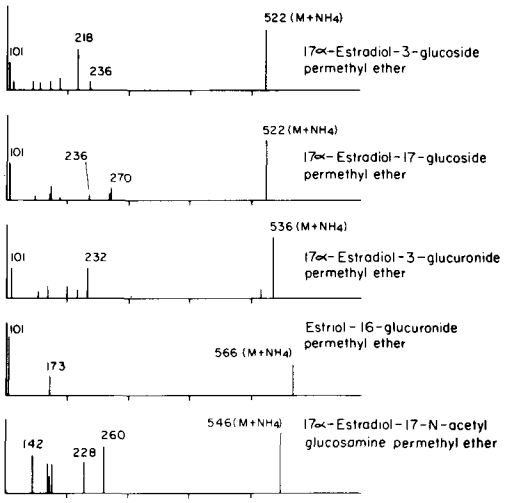

FIG. 6. NH_3 CI of steroid glycoside permethyl ethers.

Detection of abundant $M+NH_4^+$ ions by ammonia CI appears to be promising for the detection of low levels of steroid glycosides in biological samples.

CI spectra of, e.g., cardiac glycosides, digitoxin and digoxin, have been obtained without derivatization using ammonia as reagent gas (26). It is apparent that the CI process has its merits in the analysis of polar steroids or steroid conjugates. Ionization enhancement of polar molecules by exposure of samples to the ion plasma in the CI process has been described (27). The experience in our laboratory with the CI-MS of various biochemical molecules has also indicated that the CI process usually gives better ionization enhancement than the EI process.

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Identification of Sterols and Bile Acids by Computerized Gas Chromatography-Mass Spectrometry

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ABSTRACT

In mass spectrometry, sterols and bile acids form fragment ions characteristic of certain steroid structures. After separation of derivatized sterols or bile acids by the gas chromatograph and fragmentation in the mass spectrometer, data collected by the computer can be collated to provide a reconstructed gas chromatogram and a series of fragment ion current chromatograms in which the relative abundances of characteristic fragment ions are plotted vs time or scan number. Intensities of these fragment ions will be greatest and hence coincide with peak elution of unidentified sterols or bile acids. The use of known amounts of labeled appropriate sterols or bile acids permits quantitation of the identified sterol or bile acid.

The successful combination of gas chromatography with mass spectrometry (1,2) has provided in one instrument an ability to separate the components of a mixture via the gas chromatograph (GC) and to identify these components by their characteristic fragmentation patterns produced by the mass spectrometer (MS). The tedium of developing useful mass spectral data, as carried out in the mid-1960s, has been replaced with real-time imagery of mass spectra obtained during the course of acquisition of the data, resulting entirely from the successful interfacing of a minicomputer with the GC-MS system. The application of a combined data acquisition system-gas chromatograph-mass spectrometer (GC-MS-COM) to the identification of sterols and bile acids and their derivatives is the subject of this paper.

A study of the mass spectral fragmentation patterns of sterols and bile acids, generally as their derivatives, shows that 2 fundamental patterns exist: (a) characteristic fragmentation resulting from substitution in the side chain, and (b) a similar characteristic loss of substituents of the steroid nucleus prior to fragmentation of the ring system. Hydroxyl or oxo groups are frequently the substituents of the nucleus or the side chain, and in MS these groups or their derivatives are generally lost with formation of fragment ions of major or modest abundance (3-10).

In Figure 1, the mass spectra (4) of the epimeric sterols, dihydrocholesterol (5α-cholestan-3β-ol) (A) and epicoprostanol (5β-cholestan-3α-ol) (B) are compared. Each exhibits an abundant molecular ion (m/e 388) and a large ion at m/e 370 (M-18) representing the loss of water; note the ions M-72 (m/e 316), m/e 257, 233 and 215. These ions represent, respectively, fragments after loss of ring A, loss of the side chain plus the nuclear hydroxyl group, loss of the side chain plus carbons 15, 16 and 17 of ring D, and loss of the nuclear hydroxyl group, side chain and carbons 15, 16 and 17 of ring D. These spectra were determined before the coupling of the GC to an MS; many of the proposed structures of fragment ions are from early studies by Friedland et al. (3). Ryhage and Stenhagen (4) noted that the ion m/e 257 was base peak (100%) in the spectrum of methyl 12α-hydroxy-5β-cholanate. Figure 2 shows the presence of the ion m/e 255 in the spectrum of 3β,26-diacetoxy-5α-cholestan-7α-ol, a sterol with a nuclear hydroxyl and a nuclear acetoxyl group. Similarly, Ryhage and Stenhagen (4) observed that the spectrum of methyl trihydroxycoprostanate showed a prominent ion at m/e 253. Thus, a prominent ion at m/e 257 can be anticipated from any monohydroxy steroid or its derivative, an ion at m/e 255 suggests the presence of 2 such hydroxyl groups or their derivatives and an ion at m/e 253 suggests the presence of 3 such groups. Experience has shown that these conclusions are generally valid if the hydroxyl groups are removed by several carbon atoms from each other (e.g., not vicinal).

The rat and mouse produce trihydroxy bile acids without substitution in ring C, namely, β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholanic acid) (β-MC) and α-muricholic acid (3α, 6β,7α-trihydroxy-5β-cholanic acid) (α-MC). When analyzed by GC-MS as the methyl ester tris trimethylsilyl (TMS) ethers, these substances provide substantially different fragmentation patterns. Depending on the temperature of the ion source, the base peak in the spectrum of derivatized β-muricholate is the ion m/e 285 or m/e 195 (7,8) (Fig. 3). The composition of the ions was confirmed by high-resolution MS (8). One of these ions is also base peak in the spectrum of an isomer of β-MC, i.e., ω-muricho-

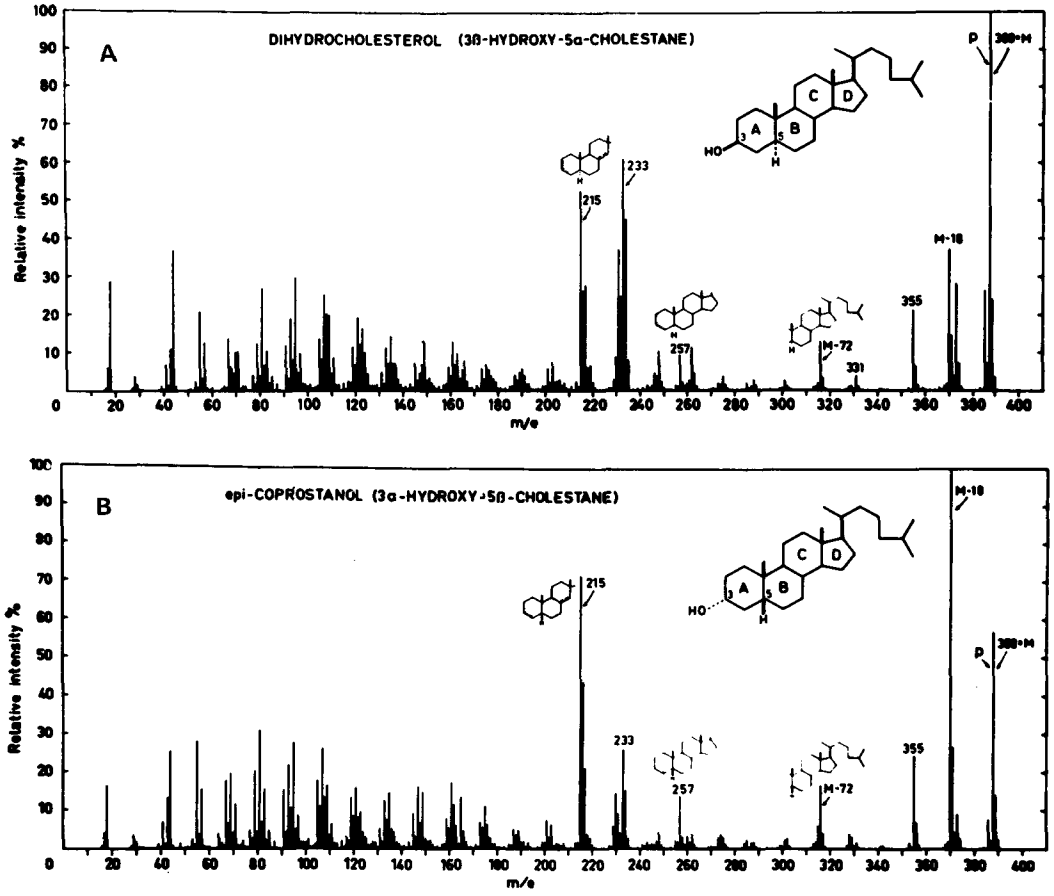

FIG. 1. Mass spectrum of (A) dihydrocholesterol and (B) epicoprostanol (4).

late, (3α,6α,7β-trihydroxy-5β-cholanic acid). However, neither of these ions is base peak in the spectrum of the TMS ether of α-MC.

Table I lists important ions and their relative abundances for 6 common bile acids derivatized as the methyl ester TMS ethers. The abundance of the base peak is underlined in each case. Note that base peak for cholate (C) is m/e 253, but this ion has only an abundance of 5% in the spectrum of α-MC, and is of no consequence in the spectrum of β-MC. Similarly, the dihydroxy acids, chenodeoxycholate (CDC) and deoxycholate (DC), which are positional isomers and are notoriously difficult to separate, provide the ions m/e 255 and 370, but with relative abundances of 16 and 100% and of 100 and 25%, respectively. The ion m/e 243 provided by CDC (7) is not normally seen in the spectrum of DC; it is found in the spectrum of C, but C and CDC derivatives are eluted form the GC column at different times. Base peak for the derivative of lithocholate (LC) is the ion m/e 372 (M-90).

After analysis of a mixture, the data in Table I can be used to ask the computer for information that is very useful in identifying particular molecules. Since the computer has acquired data from repeated scans over a mass range of the order of m/e 10-800 at a fixed rate (15-50 KHz or 3-8 sec/scan), a properly programmed system can be requested to provide a plot of the total ion current (TIC) vs relative intensity (or response). The result will be a reconstructed gas chromatogram equivalent to that obtained by the strip chart recorder. In addition, the operator can request that the system also plot fragment ion current (FIC) chromatograms (11), i.e., plots of the relative abundances of given fragment ions in each of the scans recorded. Figure 4 shows such a plot from scans numbered 110 through 170 of the abundances of all ions (the TIC) and the ions m/e 253, 368 and 458. The uppermost curve (TIC), a portion of the reconstructed gas chromatogram, shows 3 peaks. Coincident with the first small peak at the left, only the ion m/e 458 appeared. Coin-

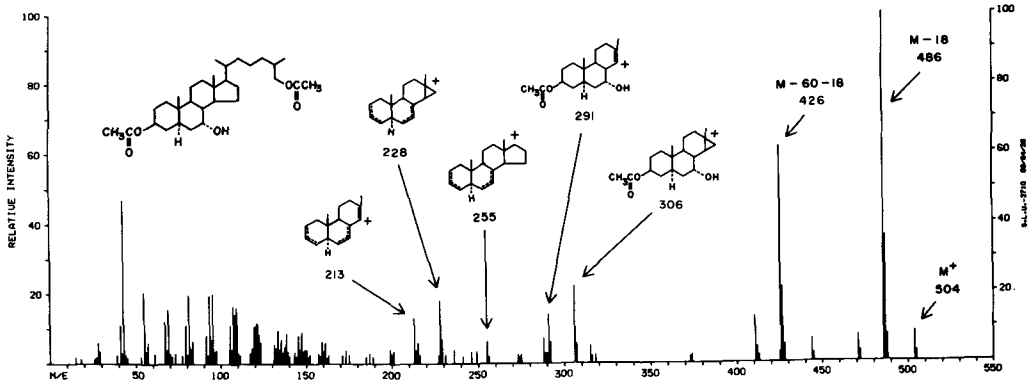

FIG. 2. Mass spectrum of 3β,26-diacetoxy-5α-cholestan-7α-ol.

cident with the large peak in the TIC plot are peaks for the 3 ions m/e 253, 368 and 458. None of these ions appeared with the third peak in the TIC plot. The size of the peak for ion m/e 458 under the second TIC peak compared to that under the first TIC peak suggests a greater abundance of this ion under the first TIC peak. In fact, the material eluted in the first TIC peak is β-MC, the second is C and the third is CDC. The identities of all peaks could be supported by replotting additional fragment ions. If necessary, a review of the full mass spectrum and comparison with that of an authentic sample, along with comparison of observed and authenticated retention times could be used to establish the identity.

An example from Sjövall's laboratory (12) of the application of this procedure to a more complex mixture is given in Figure 5. This is a plot of scan number vs 19 FIC chromatograms of important fragment ions of dihydroxy bile acids as their methyl ester TMS ethers, obtained after solvolysis of the monosulfate fraction from the urine of a patient with intrahepatic

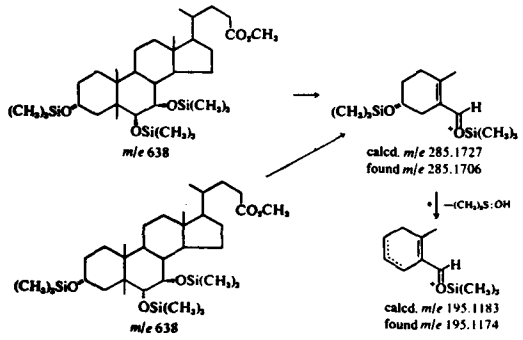

FIG. 3. Fragment ions m/e 285 and 195, derived from the TMS ether-methyl ester of β-muricholate and ω-muricholate.

cholestasis. A total of 14 different ions corresponds to elution of CDC. Seven bile acids were identified, some with small peak heights, suggesting low concentrations. Clearly, an FIC plot of many fragment ions characteristic of each substance can be very useful.

Two of the bile acids identified, allodeoxycholic (allodeoxy) and its 3β-isomer (allo-3β, 12α), (Fig. 5) are members of the 5α-series. Figure 6 shows the coplanar nucleus of a 5α- or allo bile acid, allocholic acid, compared to the structure of the more widely distributed 5β-analog, cholic acid. In GC (13), some such derivatized 3α-hydroxy allo bile acids are eluted before the corresponding 5β-analogs. Fragmentation patterns in MS are similar (7-9), but occasionally the relative abundance of certain ions is quite helpful for identification. For example, the fragment ion m/e 261 (Fig. 7) is more abundant in the spectrum of the TMS ether of methyl allocholate than that of the 5β-isomer; base peak for the former is the ion m/e 343 [M-(115+2x90)], but is the ion m/e 253 [M-(115+3x90)], for the 5β-analog. Identification of the methyl ester-TMS ether of 3α,7β,12α-trihydroxy-5α-cholantate was based (12) on the ions at m/e 458, 343 and 253.

Miyazaki et al. (14) have modified in 2 respects this GC-MS-COM procedure for determination of human biliary cholesterol and bile acids. Bile acids were converted to their ethyl esters with 5% hydrogen chloride in ethanol, and the resulting esters were treated with dimethylethylsilyl imidazole to afford the dimethylethylsilyl (DMES) ethers. The products were separated on a column packed with a 1% mixture of the phases OV-101 and Dexsil 300GC (1:1) on Gas Chrom Q at 280 C (Fig. 8). Mass spectra showed a characteristic abundant ion (M-29) for the DMES derivatives of cholesterol and of each of 5 bile acids. That this

TABLE I

Fragment Ions of Bile Acid (BA) Methyl Ester TMS Ethers, Useful for Identification by GC-MS-COM

BA	m/e (Relative abundances)
C[a]	129(11), 243(12), 253(100), 368(93), 458(72).
α-MC	129(5), 195(15), 253(5), 368(8), 443(9), 458(100).
β-MC	195(83), 285(100), 458(11), 548(11).
CDC	129(9), 243(9), 255(16), 355(19), 370(100).
DC	129(6), 255(100), 345(18), 370 (26), 535(12).
LC	129(10), 257(25), 357(27), 372(100).

[a]C = cholate; α-MC = 3α,6β,7α-trihydroxy-5β-cholanate; β-MC = 3α,6β,7β-trihydroxy-5β-cholanate; CDC = chenodeoxycholate; DC = deoxycholate; LC = lithocholate.

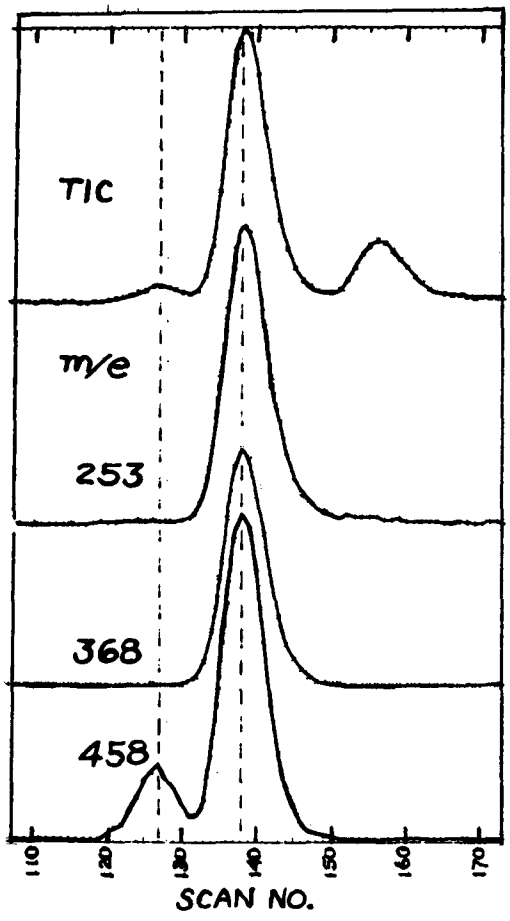

FIG. 4. Fragment ion current (FIC) chromatogram: Total Ion Current (TIC) and the ions m/e 253, 368 and 458.

fragment ion was derived from loss of mass 29 from the DMES ether and not the ethyl ester was confirmed by examination of the spectrum of the bis DMES ether of [$^2H_5$]-ethyl DC. All fragment ions which retained the ester group were 5 mass units larger than those of the undeuterated material.

Table II contains important fragment ions and their relative abundances for DMES derivatives used in these analyses. Base peak for the derivative of cholesterol and each of the bile acid ethyl esters except for CDC is the fragment ion M-29; for CDC, the base peak is the ion m/e 385 [M-(104+103)], where dimethylethylsilanol has a mass of 104. The fragment ion m/e 253 for C and m/e 255 for CDC and DC represent the unsaturated steroid nucleus, whereas the ion m/e 215 for LC represents the fragment containing the elements of rings A, B and C. The ion m/e 329 (M-143) from cholesterol represents the loss of the DMES moiety and of carbons 1, 2 and 3 of ring A. The fragment ion (M-104) is used to identify the derivatives of cholesterol (m/e 368), UDC (m/e 488) and LC (m/e 386). The ion [M-(104+29)] is used for CDC (m/e 459); the ion [M-(2x104)], m/e 384, is used for UDC and DC; and the ion m/e 383

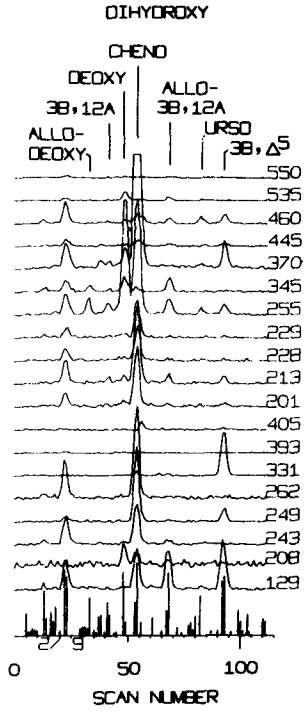

FIG. 5. Fragment ion current chromatogram of TMS ether-methyl esters of dihydroxy urinary bile acids. Fragment ions are shown (right) vs scan number (12).

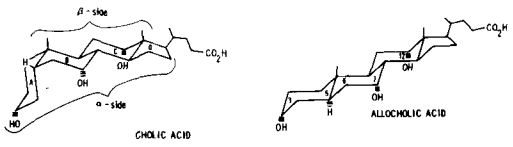

FIG. 6. Structures of allocholic and cholic acids.

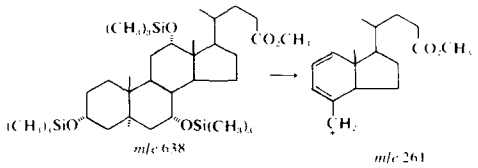

FIG. 7. Fragment ion m/e 261 derived from TMS ether-methyl ester of allocholate.

[M-(2x104+103)] is used for C. The ion m/e 143 was, in fact, base peak in the spectrum of cholesterol DMES, and is the DMES analog of the ion m/e 129 (Table I) derived from the TMS ether.

Miyazaki et al. plotted FIC chromatograms (14) with these ions (Fig. 9) and confirmed the identity of the compounds reported in Figure 8. The identity could be established by a "Computer Controlled Intensity Matching Technique" in which the size of each FIC peak

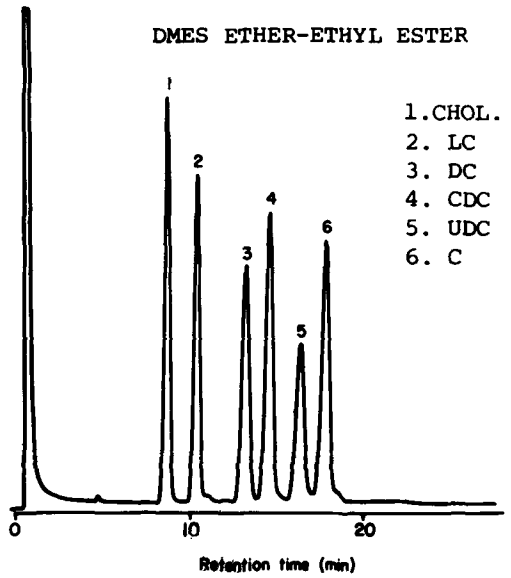

FIG. 8. Gas chromatogram of a mixture of dimethylethylsilyl ether of cholesterol (1, Chol.), the dimethylethylsilyl ether-ethyl esters of lithocholate (2,LC), deoxycholate (3, DC), chenodeoxycholate (4, CDC), ursodeoxycholate (5, UDC) and cholate (6, C) (14).

TABLE II

Fragment Ions of DMES Ethers of Steroids, Useful for Identification by GC-MS-COM

Steroid	m/e (Relative abundances)
Chol.[a]	329(66.4), 368(33.0), 443(100.0)
C	253(25.9), 383(32.7), 665(100.0)
UDC	384(20.0), 488(19.6), 563(100.0)
CDC	255(20.2), 385(100.0),459(74.0)
DC	255(65.5), 384(7.8), 563(100.0)
LC	215(52.8), 386(57.3), 461(100.0)

[a]Chol. = cholesterol; C = cholate; UDC = ursodeoxycholate; CDC = chenodeoxycholate; DC = deoxycholate; LC = lithocholate. Data from ref. 14.

was multiplied by a factor derived from the ratio of the relative abundance (%) of base peak, divided by the relative abundance of the specified fragment ion. Ideally, all 3 such fragment ions should then appear as one coincident peak. For those who prefer to carry out GC of the bile acids as the acetate-methyl esters, Szczepanik et al. (10) have published an atlas of fragment ions derived by electron impact or by chemical ionization mass spectrometry (CI-MS) of these derivatives from 48 bile acids.

Exposure of a sterol to a hepatic microsomal preparation for some period of time provides a succession of derivatives hydroxylated in the side chain as determined by GC-MS. Thus, the products of rabbit hepatic microsomal hydroxylation of 5α-cholestane-3α,7α-diol were found to include not only the 12α-ol (15), but also the

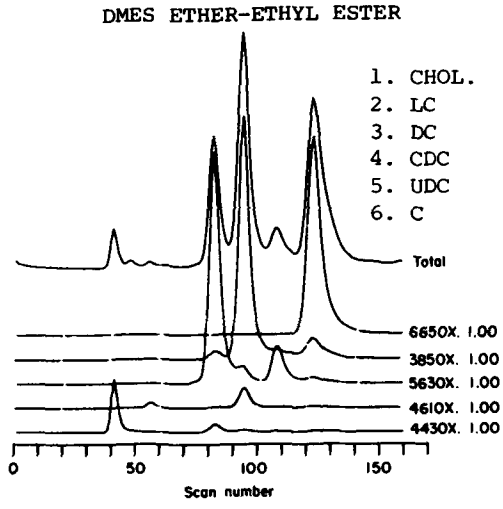

FIG. 9. Fragment ion current chromatogram of the dimethylethylsilyl ethers of the derivatives shown in Fig. 8. (14).

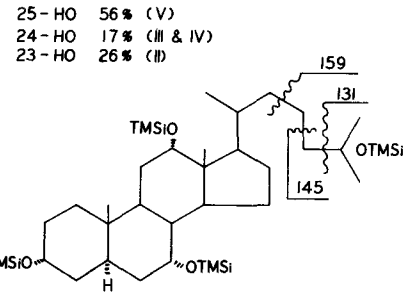

FIG. 10. Fragmentation of the side chain of the trimethylsilyl ether of 5α-cholestane-3α,7α,12α-triol, substituted at C-25 (m/e 131), C-24 (m/e 145) and C-23 (m/e 159).

tetrols with side chain hydroxylations at C-25, C-24 and C-23 (Fig. 10). Fragmentation of the tetra-TMS ethers of these derivatives provide abundant ions at m/e 131, 145 and 159. The GC record showed that both the 24R- and 24S-isomers were formed (15). Similar studies have been published for the TMS ethers of [26-$^2H_3$]-5β-cholestane-3α,7α,12α-triol and [24-$^2H_2$] cholestane-3α,7α,12α-triol (16), the 23, 24 and 25-hydroxy derivatives of 5β-cholestane-3α,7α,12α-triol (17) and a series of pentols, such as 5β-cholestane-3α,7α,12α-24ξ,26-pentol (18).

By the addition of known amounts of bile acids or sterols with stable isotopes, quantitative work can be undertaken with assurance that the computer will provide an assay of the desired fragment ions when operated in the selected ion mode (19,20) (multiple ion detection or mass fragmentography). A number of laboratories (21-25) have used deuterated bile acids such as [11,12-$^2H_2$]-CDC as the internal standard and the fragment ion m/e 372 for reference. Ahlberg et al. (24) assayed serum bile acids with added amounts of [2,2,3,4,4-$^2H_5$]-C and [11,11,12-$^2H_3$]-DC with the fragment ions m/e 368, 370 and 373 for the TMS ethers-methyl esters corresponding, respectively, to M-(3x90) of C, M-(2x90) of DC and CDC, M-(3x90) of [$^2H_5$]-C and M-(2x90) of [$^2H_3$]-DC. Recently, Karlaganis et al. (25) used [11,12-$^2H_2$]-CDC to determine 5 serum bile acids with a glass capillary column coated with barium carbonate-polyethylene glycol 20,000 (0.2%), interfaced with an MS. Extension of these studies to a larger number of epimeric bile acids and other glass capillary column installed in an LKB 9000 MS, is currently underway in our laboratory.

ACKNOWLEDGMENT

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Analysis and Structure Determination of Unsaturated 5β-Cholanoic Acids

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ABSTRACT

Unsaturated cholanoic acids are known to arise as artifacts of chemical transformation processes and during storage and high-temperature gas liquid chromatography (GLC) of various derivatives of saturated bile acids. Nevertheless, there is evidence for their natural occurrence and isolation under conditions where artifactual formation of unsaturated bile acids would be unlikely. Since structural identification of such compounds is often complicated by a lack of knowledge of their analytical properties, a representative series of monounsaturated cholanoic acids with double bonds in rings A, B and C were prepared by $POCl_3$ and $ZnCl_2$ dehydration of saturated bile acids with selectively blocked hydroxyl functions. The cholenoic acids were indistinguishable from their saturated analogs by thin layer chromatography (TLC) on plain silica gel, but those compounds with sterically exposed double bonds were resolved by $AgNO_3$-TLC, using chloroform/methanol solvent systems. The synthetic 5β-cholenoic acids obeyed the general rules of GLC mobility based on the overall shape of the molecule and the number and configuration of the functional groups. Constant retention factors attributable to the double bond were observed for all of the double bond types on several GLC phases, and theoretical retention times could be calculated for combinations of double bonds and functional groups not specifically represented among the synthetic standards. With gas chromatography-mass spectrometry (GC-MS), the unsaturated bile acids gave several characteristic fragments, which, in conjunction with the chromatographic properties of the parent compounds, permitted an unambiguous distinction among different unsaturated acids, and between unsaturated and saturated bile acids of the same number and configuration of functional groups. For complete structural identification of saturated and unsaturated bile acids, capillary GC-MS represents the ideal state of the art, but the less expensive combination of $AgNO_3$-TLC and GLC also can yield much useful information concerning the structure of natural and synthetic 5β-cholenoic acids. This study emphasizes the need for special precautions in the isolation and derivatization of bile acids intended for studies of unsaturated components.

INTRODUCTION

Unsaturated bile acids are known to occur in a wide variety of biological and experimental settings. The most commonly reported natural unsaturated bile acids are those retaining the 3β-hydroxy-Δ^5-configuration of the precursor cholesterol. Thus, the 3β-hydroxy-Δ^5-cholenoic acid is the major bile acid in the urine of healthy adults (1), and it has been suggested that it is an intermediate in a minor pathway of cholesterol metabolism. The 3β-hydroxy-Δ^5-cholenoic acid has also been found in the urine of children with extrahepatic bile duct atresia (2) and in adults with cholestatic liver disease (3). It has been suggested that this acid arises via a simple side-chain oxidation of cholesterol in response to low ATP levels in liver disease (4). The presence of 3β-hydroxy-Δ^5-cholenoic acid in human meconium (5) and in the bile and feces of newborn and fetal guinea pigs (6) has led to speculation that this minor pathway of cholesterol metabolism may predominate in the early life of man and the guinea pig. It also has been shown that the 3β-hydroxy-Δ^5-cholenoate is an intermediate in the conversion of cholesterol to lithocholate and chenodeoxycholate in both rat liver mitochondria and in the intact animal (7). Likewise, the 3β,7α-dihydroxy-Δ^5-cholenoic acid has been found to be an intermediate in the formation of chenodeoxycholic acid from 7α-cholesterol in rat liver mitochondria (8) and in intact rats (9). Evidence for an analogous pathway in the hen (10,11) and the carp (12) has also been obtained.

Furthermore, unsaturated bile acids are known to be involved as intermediates in the bioconversion of primary to secondary bile acids. This has been convincingly demonstrated in the rat and the rabbit, using doubly labeled cholic acid. From the label remaining in the recovered deoxycholic acid, it was deduced that the intestinal degradation of cholic acid involved a dehydration at the 7α-position, giving rise to a 3α,12α-dihydroxy-Δ^6-cholenoic acid intermediate (13). It was subsequently shown that a synthetic radioactive 3α,12α-dihydroxy-Δ^6-cholenoic acid was converted to deoxycholic acid in the rat intestine (14). Likewise, a human fecal bacterium has been found to dehydrate cholic acid at the 7α-position and to reduce synthetic 3α,12α-dihydroxy-Δ^6-cholenoic acid to deoxycholic acid (15). A major

pathway in the bacterial modification of bile acids leading to unsaturation of the steroid nucleus involves a prior oxidation of the hydroxyl group to a keto group. *Escherichia coli* from human feces have been found to convert cholic, chenodeoxycholic, deoxycholic and lithocholic acids to the 1,4-dien-3-one derivatives with a variety of degraded side chains (16). Similarly, a set of 4 unsaturated intermediates, leading to the aromatization of rings A and B, have been described using human gut bacteria (17-19). Finally, incubation of lithocholate-3-sulfate with fecal microorganisms was found to give rise to Δ^2- and Δ^3-cholenoic acids (20,21). Thus, natural samples of bile acids can contain small amounts of unsaturated homologs (1,22), while unsaturated bile acids in relatively large amounts have been found in the hepato-biliary extracts of a 3200-year-old Egyptian mummy (23).

Unsaturated bile acids, corresponding to the loss of one or more hydroxyl groups at C_3, C_6, C_7 and C_{12} are known to arise as artifacts of dehydration of saturated bile acids during chemical derivatization (24,25) and during storage and high-temperature gas liquid chromatography (GLC) of the trifluoroacetyl (26,27) and possibly trimethylsilyl (28,29) derivatives. In addition, saponification of the 3-, 7- and 12-sulfate esters of saturated bile acids leads to formation of corresponding unsaturated bile acids (20,25,30,31).

In the past, the identification of both chemical and biochemical desaturation products of bile acids has been complicated by a lack of knowledge of their analytical properties and the unavailability of reference standards. We have recently prepared a representative series of monounsaturated, mono- and disubstituted cholanoic acids and have examined their chromatographic and gas chromatographic-mass spectrometric (GC-MS) properties (32,33). In this communication, we have reviewed this work along with the evidence for the artifactual formation of unsaturated bile acids during common analytical transformations.

EXPERIMENTAL PROCEDURES

The monounsaturated 5β-cholanoic acids with double bonds in rings A, B and C (Fig. 1) were prepared by $POCl_3$ and $ZnCl_2$ dehydration of saturated bile acids with selectively blocked hydroxyl functions, as previously described (32). The unsaturated ketocholanoic acids were gifts from Drs. B.A. Gordon and P. Ziegler. The 3α,12α-diacetoxy-$\Delta^{9(11)}$- and 3α,12β-diacetoxy-$\Delta^{9(11)}$-cholenoic acids were obtained from Steraloids Co., Pawling, NY.

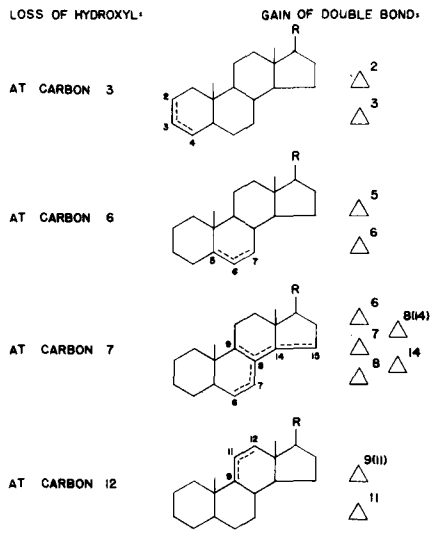

FIG. 1. Structure and nomenclature of the unsaturated bile acids prepared in the laboratory.

The acids were converted into the methyl esters by reaction with dimethoxypropane-HCl (34) and were acetylated by acetic anhydride/perchloric acid (35). The trifluoroacetates of saturated bile acid methyl esters were prepared at 40 C (40 min) in the presence of trifluoroacetic anhydride, as previously described (36).

For thin layer chromatography (TLC) on plain silica gel, SIL-G-25 (0.25 mm) plates (Brinkmann Industries, Westbury, NY) were used. $AgNO_3$-TLC was performed on Uniplates (20% $AgNO_3$, 0.25 mm layer), coated with Silica Gel G (Analtech, Newark, DE). The plates were activated for 1 hr at 125 C prior to spotting and were developed in chloroform/methanol (200:1) (33). The separated compounds were visualized by a 2′,7′-dichlorofluorescein spray. The compounds were recovered from the silica gel by elution with chloroform/methanol (2:1).

GLC separations of bile acid methyl ester acetates were carried out isothermally on glass columns (90-180 cm × 0.3 cm id), using 1-3% OV-225 (cyanopropylphenylsiloxane), SE-30 (methylsiloxane) and Poly S-179 (polyphenyl ether sulfone) on 100-120-mesh Gas Chrom Q as the column packings (Applied Science Laboratories, State College, PA). The columns were installed in either an F&M Biomedical Gas Chromatograph (F&M Scientific Corp., Avondale, PA) or in a Varian 2400 Gas Chromatograph (Varian Associates, Palo Alto, CA), equipped with flame-ionization detectors. The carrier gas was nitrogen at a flow rate of 20 ml/min. Detailed operating conditions are given in

the legends to the appropriate figures.

Combined GC-MS of the bile acid methyl ester acetates or trifluoroacetates was performed as previously described (32), using a Varian MAT CH-5 single-focusing instrument, coupled to a Varian Model 2400 gas chromatograph. The acetates were analyzed on a 1% SE-30 column (90 cm × 0.3 cm id), whereas a 1% OV-210 (a fluoroalkylsiloxane) column was used for the trifluoroacetates. In both instances, helium was the carrier gas. Detailed analytical conditions are given in the legends to the appropriate figures. A limited number of analyses with the methyl ester acetates were made by capillary GC-MS using the Hewlett-Packard 5985A automated instrument, equipped with a 10-m flexible fused silica capillary column, coated with a Carbowax-OV-1 film. The samples were introduced by direct injections, using helium as the carrier gas. The capillary column was temperature-programmed in the range 250-300 C.

RESULTS AND DISCUSSION

A reliable identification of unsaturated bile acids in natural extracts depends on a successful separation of the saturated and unsaturated components. Since certain saturated and unsaturated bile acids may overlap in any one chromatographic system or may yield comparable spectra in the mass spectrometer, usually 2 or more analytical systems may need to be combined to obtain an unambiguous result.

Analysis and Structure of Synthetic Standards

Of the micropreparative methods of potential interest to the isolation of unsaturated bile acids, TLC, GLC and LLC (liquid-liquid chromatography) may be considered. There have been no reports of the application of LLC to the separation of saturated and unsaturated bile acids.

TLC

An effective technique for the resolution of saturated and unsaturated steroids is $AgNO_3$-TLC (37,38). The location of the olefinic site in the ring system greatly influences the polarity of the double bond and its ability to form π-complexes with the silver cations in the silica gel. Only a few applications of this technique have been made to bile acids (39,40). Figure 2 summarizes our results on the TLC behavior of the saturated and monounsaturated bile acid methyl ester acetates on silver nitrate-treated silica gel. The chloroform/methanol solvent system was found to separate the bile

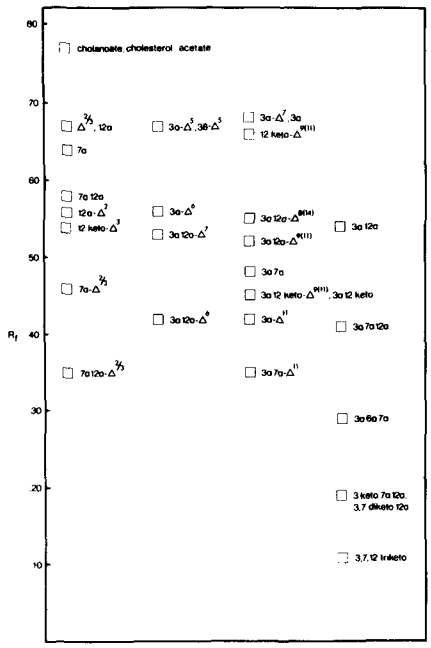

FIG. 2. Schematic survey of the R_f values of saturated and unsaturated bile acids on $AgNO_3$-TLC. Adsorbent: 20% $AgNO_3$-Silica Gel G, 0.25-mm layer (Uniplates, Analtech); solvent system: chloroform/methanol (200:1); development time: 30 min.

acid methyl ester acetates according to the number and position of the acetoxy or ketone groups. Tables of detailed numerical R_f values have been published elsewhere (34,41). The methyl ester acetates of the unsaturated bile acids migrate with their saturated counterparts on plain silica gel, but on the $AgNO_3$-treated gel, the migration of the Δ^2-, Δ^3-, Δ^6 and Δ^{11}-cholenoates is found to be retarded in relation to that of their saturated analogs. The highest degree of interaction with the silver ions is noted for the ring A unsaturated diacetoxy cholenoates and with the monoacetoxy-Δ^{11}-cholenoate, which is completely resolved from the isomeric Δ^6-cholenoate. Separation of the monoacetoxy-Δ^6-5β-cholenoate from the corresponding saturated analog has been reported for the free acids (39). Only the Δ^{11}-monohydroxy and -dihydroxy bile acids have been previously resolved from their saturated analogs as the methyl ester acetates (40). The resolution is probably based on the disubstituted nature of these double bonds (no interaction with ring junctions) and their occurrence in exposed positions on the steroid ring system allowing π-bonding with the silver ions in the silica gel. Similar effects of double bond location have been noted for the unsaturated

sterols, where the importance of masking of nuclear (37) and side-chain (38) double bonds by an adjacent alkyl group was also evident. We have already pointed out (33) that the relative ease of complexing of the bile acid double bonds complexing with the silver ions, as evidenced by the relative migration rates, parallels the susceptibility of the bile acids to hydrogenation by methods dependent on π-complex formation.

Similar separations of bile acid methyl ester acetates have been obtained with a benzene/ethyl acetate solvent system (23). However, the toxicity of benzene and its capacity to quench the fluorescence of 2',7'-dichlorofluorescein during visualization of the TLC plates has made it desirable to replace the benzene-containing solvents by chloroform/methanol. However, the separation of isomeric unsaturated bile acids within a class of functional groups with either of the solvent systems is limited and other methods must be found for preparation of pure isomers.

GLC

The bile acid methyl ester acetates which overlap or remain poorly resolved on $AgNO_3$-TLC may be further separated by GLC to give essentially complete resolution of all common saturated and unsaturated 5β-cholanoic acids. Table I gives the relative retention times of the various 5β-cholenoic acids on 3 liquid phases selected for their high resolution (42) of configurational isomers (OV-225) and for their general applicability to GC-MS (43) of the bile acid methyl ester acetates (SE-30 and Poly S-179). From the relative retention times, it is seen that separation of the configurational isomers of the 5β-cholenoic acids with 1-2 acetoxy groups is achieved in a manner similar to that obtained for the corresponding saturated acids (41-43). A useful measure of the extent of the resolution is provided by the ratio of the relative retention times of the unsaturated compound and its saturated analog. A value differing by more than 0.10 from unity generally indicates a baseline resolution of any 2 components (33,41).

The predominant factor contributing to the differences in the GLC behavior of the unsaturated bile acids appears to be the ability of the double bond to alter the orientation of the acetoxy group at C_3 through the interaction with the A/B ring junction. This is best seen in the behavior of the 3 3α-acetoxy-5β-cholenoic acids with double bonds in ring B (Fig. 3). The Δ^5-bond flattens the molecule at the A/B ring junction causing an increase in the interaction with the liquid phase and (or) support, leading to an increase in relative retention time similar

TABLE I

Relative Retention Times of 5β-Cholenoic Acid Methyl Ester Acetates on OV-225, SE-30 and Poly-S-179 Liquid Phases

Component	Retention time relative to saturated analog[a]		
	OV-225	SE-30	Poly-S-179
Δ^3-Cholenoic	1.000	0.668	1.032
Δ^2-Cholenoic	1.070	0.988	1.146
12α-OH-Δ^3	1.038	0.948	1.063
7α-OH-Δ^3	1.020	0.937	1.056
7α-OH-Δ^2	1.123	1.000	1.168
12-Keto-Δ^3	0.992	0.927	1.000
7α,12α-diOH-Δ^3/Δ^2	1.053	0.959	1.110
3α-OH-Δ^5	1.179	1.113	1.167
3β-OH-Δ^5	1.204	1.156	1.283
3α-OH-Δ^6	0.917	0.918	0.927
3α,12α-diOH-Δ^6	0.941	0.922	0.913
3α-OH-Δ^7	1.033	1.015	1.048
3α,12α-OH-Δ^7	1.016	0.986	1.075
12-Keto-$\Delta^{9(11)}$	1.185	1.029	1.155
3α,12α-diOH-$\Delta^{9(11)}$	0.869	0.879	0.860[b]
3α,12β-diOH-$\Delta^{9(11)}$	0.940	0.848	0.851[b]
3α-OH,12-Keto-$\Delta^{9(11)}$	1.117	0.992	1.130[c]
3α,12α-diOH-$\Delta^{8(14)}$	0.959	0.940	0.968[c]
3α-OH-Δ^{11}	0.965	0.956	0.972
3α,7α-diOH-Δ^{11}	0.977	0.987	0.979[c]

[a]With methyl deoxycholate diacetate at 15 min.

[b]Recorded at 240 C; flow rate 20 ml/min.

[c]Have been previously reported (43).

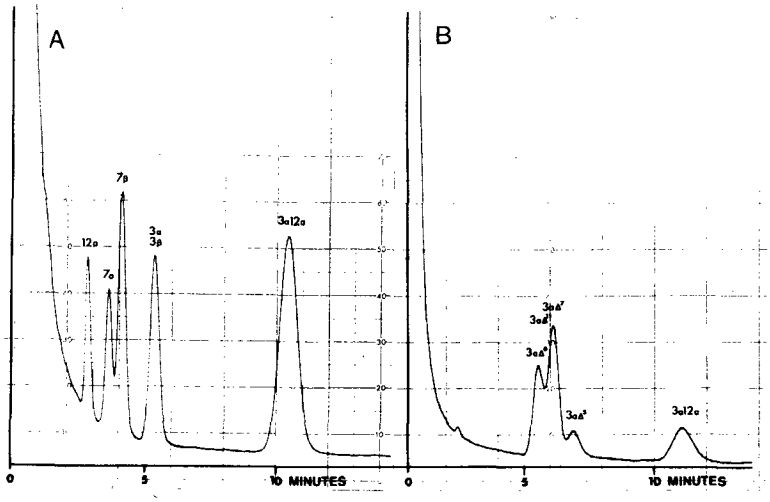

FIG. 3. GLC of saturated (A) and unsaturated (B) monoacetoxy-5β-cholanoic acid methyl esters. The cholanoic acids are identified by the position and orientation of the acetoxy group and by the location of the double bond; methyl 3α,12α-diacetoxy-5β-cholanoate internal standard. Column: 1% OV-225 on Gas Chrom Q (100-120 mesh) in a 90 cm × 2-mm id glass U-tube. Column temperatures: 255 C (A); 250 C (B), isothermal. Sample: 0.5 μl of ca. 1% solution in chloroform.

to that observed for the 5α-cholanoic acids (42,43). The magnitude of shift in the retention time values of the unsaturated compounds decreases upon progressing from Δ^5- through Δ^6- to Δ^7-acids, reflecting the gradual displacement of the double bonds from the vicinity of the A/B ring junction. Of the ring B unsaturates, only the Δ^5-compounds show baseline resolution from the saturated analogs on each of the liquid phases. The Δ^6-5β-cholenoic acids appear as leading shoulders on the peaks of the corresponding saturated acids, whereas the Δ^7-compounds overlap completely with their saturated analogs. The Δ^6- and Δ^7-derivatives are partially resolved from each other, and, in the case of the monoacetoxy derivatives, they are also resolved from the Δ^5-isomers. The order of elution of the ring B unsaturates was found to be the same for all 3 liquid phases: $\Delta^6 > \Delta^7 > \Delta^5$. Surprisingly, this trend does not appear to hold for the methyl ester trifluoroacetates of the 5β-cholenoates with ring B unsaturation, which are not resolved from the saturated analogs on any of a variety of liquid phases (29,44).

Of the ring C unsaturates, the $\Delta^{8(14)}$-derivative shows poor resolution from the saturated analog as well as from the Δ^7-isomer on all 3 liquid phases examined. However, effective resolution can be obtained with capillary columns. Comparable difficulties in the resolution of the Δ^7- and $\Delta^{8(14)}$-compounds have been experienced with the 5β-sterols, although the corresponding derivatives of the 5α-sterols have been resolved on 4 different types of liquid phases, including SE-30 (45). The Δ^7- and $\Delta^{8(14)}$-cholenoates are also known to be poorly resolved as the methyl ester trifluoroacetates (29), but resolution of the 3α,12α-ditrifluoroacetoxy-$\Delta^{8(14)}$-5β-cholenoate and its saturated analog has been achieved on SE-30 (29) and on a variety of other liquid phases (44). The separation of the 3α,12α-diacetoxy-$\Delta^{8(14)}$-5β-cholenoate and its saturated analog has been reported on SP-525 (46), but problems with stability of this aromatic hydrocarbon phase have caused it to be withdrawn by the manufacturer (43). However, the Poly S-179 phase, intended as its replacement, is not able to resolve the 2 compounds (43). The Δ^7- and $\Delta^{8(14)}$-isomers are readily resolved by capillary GLC on nonpolar liquid phases (results not shown). In contrast, the $\Delta^{9(11)}$-cholenoates are well separated from their fully saturated counterparts. The epimeric 12-acetoxy-$\Delta^{9(11)}$-compounds are well resolved from each other and from the saturated analogs on each of the liquid phases, reflecting a radical alteration of the configuration of the 12-acetoxy group by the adjacent olefinic site (Fig. 4). Similar behavior was observed for the 12-keto-$\Delta^{9(11)}$-5β-cholenoates, except that their resolution from the corresponding saturated compounds was lost on SE-30 phase. The 12-acetoxy-$\Delta^{9(11)}$-cholenoic acids showed a partial degradation at the usual operating temperatures of the silicone-based liquid phase, with complete destruction of the compounds at

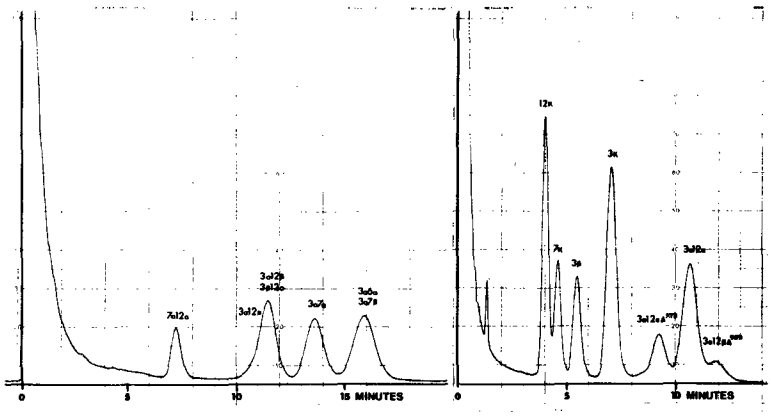

FIG. 4. GLC of saturated (A) and unsaturated (B) diacetoxy-5β-cholanoic acid methyl esters. The cholanoic acids are identified by the position and conformation of the acetoxy groups and by the location of the double bond. Column: as in Fig. 3. Column temperature: 255 C, isothermal. Other conditions as in Fig. 3.

the higher temperatures used with the Poly S-179 columns. It is notable that, on the OV-225 liquid phase, each of the 12-keto-$\Delta^{9(11)}$-cholenoic acids assayed was eluted in reverse order to that of the corresponding acetoxy derivatives when compared to the saturated counterparts. Thus, the methyl 12-keto-$\Delta^{9(11)}$-cholenoate was eluted after the saturated analog, whereas the 12-acetoxy-$\Delta^{9(11)}$-cholenoate was eluted ahead of the saturated analog (Figs. 4 and 5). The effect of the $\Delta^{9(11)}$-bond is thought to be a reduction of the molecular volume of the hydroxylated bile acid derivative (46). An examination of the Dreiding model suggests that this effect is not realized in the 12-keto-$\Delta^{9(11)}$-cholenoic acid, explaining the difference in the elution order. It is possible that other 5β-cholenoic acids also undergo olefin-induced changes in their molecular volumes that are consistent with the alterations in their retention times. This possibility may offer an alternative to changes in polarity as an explanation for the observed retention effects.

Of the ring A unsaturates, the Δ^2-isomer is retained longer than the Δ^3-isomer, which overlaps with the corresponding saturated compound on OV-225 and on Poly S-179. On SE-30, the order of elution of the Δ^2- and Δ^3-isomers remains the same as on the polar

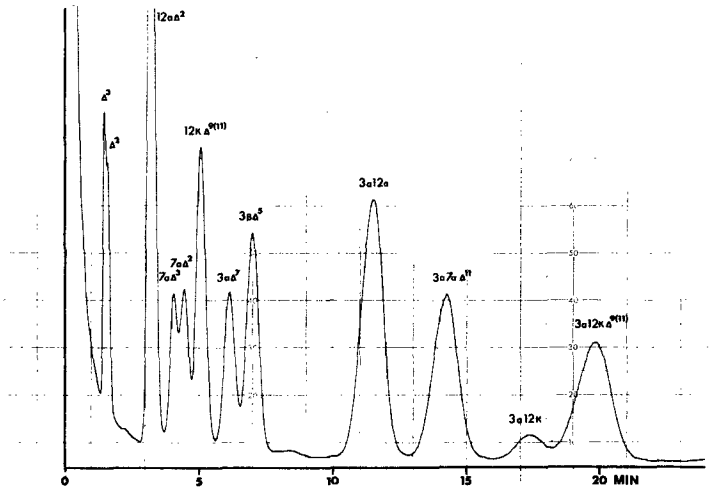

FIG. 5. GLC of unsaturated 5β-cholanoic and ketocholanoic acid methyl esters. The cholanoic acids are identified by the position and orientation of the functional groups and by the location of the double bond. Column and operating conditions as in Fig. 3.

liquid phases, but the saturated acid now overlaps with the Δ^2-isomer. Figure 5 shows the separation of Δ^2- and Δ^3-cholenoic acids on OV-225. A complete resolution of these compounds requires ca. 3000 theoretical plates, readily obtained on a good 180-cm packed column. Equally effective separations are obtained for the Δ^2- and Δ^3-isomers of 7α-hydroxycholanoic acid.

Table II gives the fractional increments in retention time characteristic of each functional group or structural feature of the saturated and unsaturated bile acid molecules. These factors may be used to estimate the relative retention times of unknown bile acids by summing the values for the predicted functional groups and that for the methyl cholanoate backbone, and converting the values to the antilogarithms. The number thus obtained is an estimate of the retention time relative to methyl deoxycholate acetate.

$AgNO_3$-TLC-GLC

The resolution of the saturated and unsaturated 5β-cholanoic acids as the methyl ester acetates can be greatly improved, and the certainty of identification of all peaks increased by a combination of $AgNO_3$-TLC with GLC. Of the 3 liquid phases, the OV-225 offers the best prospects, although it cannot be readily used for GC-MS (43). The combination of $AgNO_3$-TLC and GLC on OV-225 has been successfully employed in the isolation and identification of the unsaturated bile acids in the hepato-biliary tissue extracts of an Egyptian mummy (23), in the isolation and purification of unsaturated bile acids prepared by chemical synthesis (32) and in the isolation and identification of the degradation products from the trifluoroacetyl derivatives of the common bile acid methyl esters (47). However, the assistance of capillary GLC is necessary for the differentiation between the Δ^7- and $\Delta^{8(14)}$-cholenoates. Thus, the methodology based on $AgNO_3$-TLC and GLC offers the potential for a systematic identification of the unsaturated bile acids in natural bile acid extracts from any source.

GC-MS

The assistance of GC-MS is necessary for the verification of the structures of any unknown bile acids and for the determination of the molecular weight and the number and nature of

TABLE II

The Effect of Various Substituents on the GLC Retention Behavior of Bile Acid Methyl Ester Acetates

Substituent	Conformation	Additivity factor[a]	Number of compounds tested
5β-Cholanoate		-0.900	
3 Keto		0.694	4
3β Ac	Axial	0.625	3
3α Ac	Equatorial	0.605	7
6 Keto		0.554	2
7 Keto		0.497	4
7β Ac	Equatorial	0.481	2
6α Ac[b]	Equatorial	0.469	1
12 Keto		0.417	4
7α Ac[c]	Axial	0.382	8
12β Ac	Equatorial	0.369	1
12α Ac	Axial	0.280	6
A/B = 5α[d]		0.085	7
Δ^5		0.076	2
Δ^2		0.031	4
Δ^7		0.010	2
Δ^3		0.008	2
Δ^{11}		-0.013	2
$\Delta^{8(14)}$		-0.018	1
Δ^6		-0.032	2
C-23-*nor*		-0.145	3

[a]The antilog of the sum of the predicted factors plus that of the methyl cholanoate backbone gives an estimate of the RRT_{deoxy} value on OV-225.

[b]6α Ac with no adjacent acetate (position 7).

[c]7α Ac with no adjacent acetate (position 6).

[d]Sterols included in the calculation.

the functional groups, including the location of the double bonds. In general, all the unsaturated compounds exhibit the major ions expected on the basis of the known behavior of the saturated 5β-cholanoic acid methyl ester acetates (48), and the majority of the fragments have been identified on this basis. In all instances, monounsaturation was recognized primarily by the mass of the nuclear ion fragment appearing at 2 mass units less than that of the corresponding saturated compounds. There were several diagnostically significant fragments that allowed distinction to be made among different unsaturated acids and between saturated and unsaturated acids of the same number and configuration of functional groups. The ions of greatest diagnostic value in the identification of the Δ^2-compounds are the retro-Diels-Alder (RDA) ions (nucleus + side chain -54, nucleus -54). These ions have been reported to be characteristic of Δ^2-steroids (49,50), of many 3-hydroxylated bile acids (49,51), and of the 3-keto-5β-cholanoic acids (49,52), which form Δ^2-en-3-ol structures through enolization. In contrast, the Δ^3-cholenoate is characterized by the absence of the RDA fragments. Thus, the RDA ions are absent from the spectrum of methyl 12-keto-Δ^3-cholenoate (32). Figure 6 compares the spectra of the Δ^2- and Δ^3-cholenoates and points out the absence of the RDA ions (m/e 203 and 318) in the spectrum of the Δ^3-cholenoate.

In a manner analogous to that observed for the saturated bile acids, the ring A unsaturates with substituents at C-12 show a marked tendency to lose the side chain (49,51,52), giving a nuclear ion fragment as the base peak. The ring A monounsaturates with substituents at C-7 give small amounts of m/e 249, representing ions arising from rings C + D + side chain, as has been previously noted for the saturated 5β-cholanoates (48). The Δ^6- and Δ^7-dihydroxycholenoates also can be distinguished from each other because the ring B cleavage products at m/e 247 occur only in the spectrum of the Δ^6-compounds, as shown in Figure 7. This ion is markedly reduced in intensity in the spectrum of the dihydroxy compounds when compared to the monohydroxylated compounds. This is probably because of the presence of the 12α-acetoxy group, which facilitates the loss of the side chain, thus avoiding the pathway to this fragment. In other aspects, the mass spectra of these compounds are similar to that of methyl triacetoxycholate. The m/e 249 is of diagnostic significance in that it indicates the potential presence of a 5,6- or 6,7-double bond, or substitution at C-6 or C-7 (14,30,53). Thus, the m/e 249 fragment is prominent in the spectra

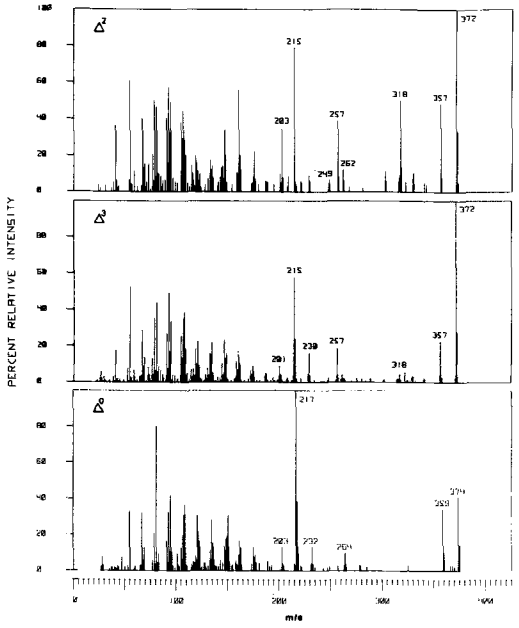

FIG. 6. GC-MS spectra of ring A unsaturated methyl cholenoates. (A) methyl Δ^2-cholenoate; (B) methyl Δ^3-cholenoate; C, methyl cholanoate. GC-MS conditions as in ref. 32.

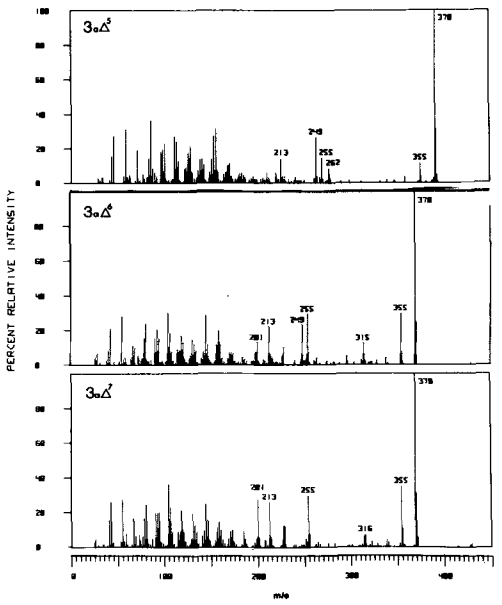

FIG. 7. GC-MS spectra of ring B unsaturated 3α-acetoxy-5β-cholanoic acid methyl esters. The cholanoic acids are identified by the location of the double bond. GC-MS conditions as in ref. 32.

of the 3-acetoxy-Δ^5-cholenoates, which yield identical ions for both epimers, as previously noted for the 3-trifluoroacetoxy derivatives (51). The presence of the m/e 262 ion in these spectra can be used to distinguish the monoacetoxy-Δ^5-from the closely similar Δ^6-compounds, which do not contain it (Fig. 7). The m/e 262 arises by ring B cleavage (51). The 6,7-double bond prohibits the cleavage of the 6,7- and 9,10-bonds necessary for the formation of the m/e 262 fragment. The fragmentation patterns of the methyl 3α-acetoxy-Δ^7-cholenoates differ from those of the isomeric Δ^5- and Δ^6-unsaturated bile acids by the absence of ions at m/e 249 and 262. The m/e 262 ion is found also with 7α-acetoxy bile acids (51), including the spectra of methyl Δ^2- and Δ^3-7α-acetoxy-cholenoates. As shown in Figure 7, the Δ^7-acid can be distinguished from the Δ^5- and Δ^6-acids on this basis, despite the lack of characteristic ions of its own.

The presence of an ion at m/e 286 is characteristic of ring C monounsaturated diacetoxy methyl cholenoates, and it occurs with greatest intensity in the spectrum of methyl diacetoxy-$\Delta^{8(14)}$-cholenoates. Otherwise these dihydroxy-cholenoates yield spectra closely similar to that of cholic acid (Fig. 8). An ion of possible diagnostic significance in the spectra of the dihydroxy-$\Delta^{9(11)}$-cholenoic acids occurs at m/e 386. It is believed to correspond to a loss of the acetate at C-3 and a loss of ketene at C-12. The mass of the ion corresponds to that of the nucleus + side chain fragment (m/e 368) with a molecule of water added (32). The diacetoxy $\Delta^{9(11)}$-compounds gave spectra differing from that of cholic acid, mostly in the intensity of the high-mass ions. This difference reflects the difficulty in removing the acetoxy group at C-12 as a result of the adjacent double bond. Of the remaining compounds with a single double bond in ring C, the Δ^{11}-cholenoic acids gave mass spectra exhibiting the expected ions, which nevertheless permitted the identification of this class of cholenoates based on their tendency to lose the side chain during fragmentation in the absence of a 12α-acetoxy function.

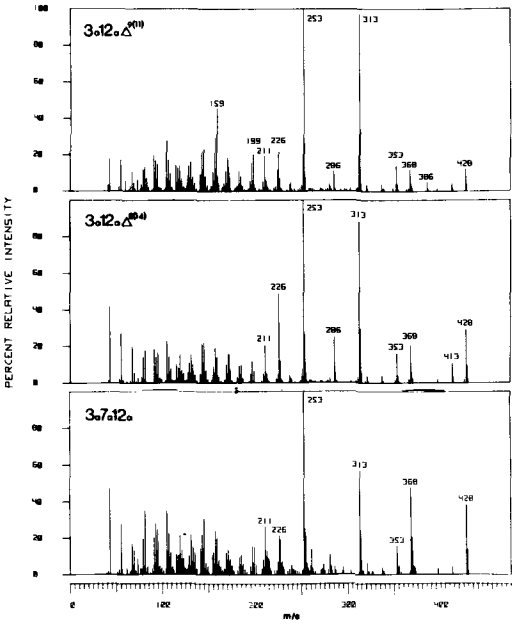

FIG. 8. GC-MS spectra of ring C unsaturated 3α,12α-diacetoxy-5β-cholanoic acid methyl esters. The cholanoic acids are identified by the location of the double bond. GC-MS conditions as in ref. 32.

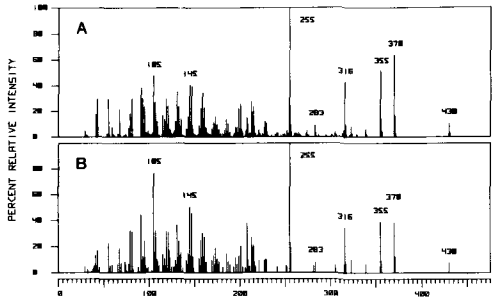

FIG. 9. GC-MS spectrum of a monounsaturated 12α-acetoxy bile acid methyl ester (A) and of a synthetic methyl 12α-acetoxy-Δ^2(Δ^3)-5β-cholenoate (B). GC-MS conditions as in ref. 23.

Analysis and Structure of Degradation Artifacts

We have employed the methodology just described for the isolation and identification of several unsaturated bile acids in the hepatobiliary tissue extracts of an Egyptian mummy (23). Figure 9 shows the GC-MS spectrum of a previously unidentified monounsaturated monohydroxy bile acid isolated from the mummy and of the corresponding spectrum of synthetic 12α-acetoxy-Δ^2(Δ^3)-cholenoate. The GC-MS spectrum of a previously unidentified monounsaturated dihydroxycholenoate isolated from the mummy bile and of the corresponding synthetic 3α,12α-diacetoxy-Δ^6-cholenoate are shown in Figure 10. These compounds showed TLC migration rates consistent with those of monoacetoxy and a diacetoxy methyl cholenoate, respectively, as well as GLC retention times compatible with the postulated 12α-hydroxy- and 3α,12α-dihydroxycholenoate structures.

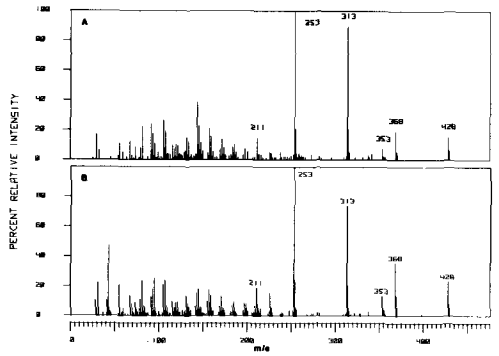

FIG. 10. GC-MS spectrum of a monounsaturated diacetoxy bile acid methyl ester (A) and of synthetic methyl 3α,12α-diacetoxy-Δ^6-5β-cholenoate. GC-MS conditions as in ref. 23.

An extensive dehydration of saturated bile acids may take place during trifluoroacetylation, as well as during storage and during high-temperature GLC of the trifluoroacetates. Figure 11 shows the total ion current plot of a gas chromatogram of the trifluoroacetylation products of methyl cholate obtained at an elevated injector temperature (47). In addition to the major peak (peak 7), which represents the anticipated methyl ester tri-trifluoroacetate of cholic acid, there are 6 additional, well resolved minor peaks, which are eluted earlier. On the basis of the GLC retention times of these peaks and of their mass spectra, we have made tentative identifications of them. In order of decreasing retention time, the extraneous peaks were identified as the methyl ester of the 3α,12α-ditrifluoroacetyl derivative of deoxycholic acid (peak 6), which was apparently present as a minor contaminant in the cholic acid preparation used for the test. Peak 5 represents the major degradation product of cholic acid. On the basis of its mass spectrum, it was identified as the methyl ester of 3α,12α-ditrifluoroacetoxy $\Delta^{8(14)}$-cholenoate, which resulted from the cholic acid by loss of the 7α-hydroxyl group (Fig. 12). Its GLC retention time and mass spectrum correspond to those reported by Kallner (53) for an authentic synthetic compound. This compound recently has been observed as the major dehydration product of cholic acid in the hepatobiliary tissue extracts from a 3200-year-old Egyptian mummy (32). A reasonable spectral match was also obtained for a minor degradation product of cholic acid (peak 4). The presence of an ion at m/e 340 suggests a ring C unsaturation, whereas the GLC retention time corresponds to a 3β,12α-di-trifluoroacetyl cholanoic acid with a double bond at $\Delta^{8(14)}$.

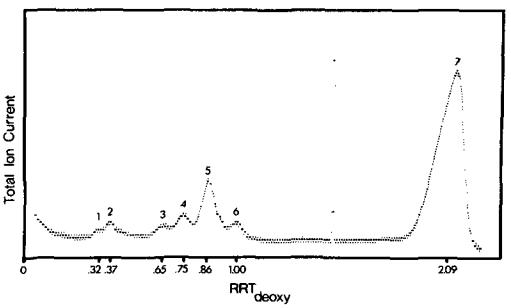

FIG. 11. GLC of methyl 3α,7α,12α-tritrifluoroacetoxy-5β-cholanote (cholate) at elevated flash heater temperature (250 C). Tentative peak identity: 1, 12α-trifluoroacetoxy-Δ^3,Δ^7-5β-choladienoate; 2, methyl 12α-trifluoroacetoxy-Δ^2,Δ^7-5β-choladienoate; 3, methyl 3α-trifluoroacetoxy-Δ^7,Δ^{11}-5β-choladienoate; 4, methyl 3β,12α-ditrifluoroacetoxy-$\Delta^{8(14)}$-5β-cholenoate; 5, methyl 3α,12α-ditrifluoroacetoxy-$\Delta^{8(14)}$-5β-cholenoate; 6, methyl 3α,12α-ditrifluoroacetoxy-5β-cholanoate (deoxycholate contaminant in test cholate). Column: 1% OV-210 on Gas Chrom Q (100-120 mesh) in a 90 cm × 2.0-mm id glass coil. Column temperatures: 225 C, isothermal. Sample size: 1 μl of ca. 1% solution in diethyl ether.

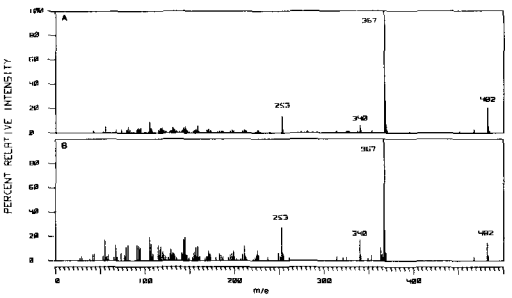

FIG. 12. GC-MS spectrum of a monounsaturated methyl 3α,12α-ditrifluoroacetoxy-5β-cholanoate (A) and of corresponding synthetic methyl 3α,12α-ditrifluoroacetoxy-$\Delta^{8(14)}$-5β-cholenoate (B). GC-MS conditions as in ref. 23.

Otherwise, the mass spectrum of this compound was similar to that of the 3α,12α-ditrifluoroacetoxy-$\Delta^{8(14)}$-cholenoate emerging behind it (peak 5) from the GLC column. To our knowledge, there is no precedent for an epimerization of hydroxyl groups during GLC degradation of hydroxy steroids. Peak 3 was identified as a monotrifluoroacetyl derivative of a diunsaturated dehydration product of cholic acid. It was eluted with a retention time corresponding to lithocholic acid, which suggested the presence of a 3α-hydroxyl group. The mass spectrum of this compound was found to have a striking resemblance to that of a synthetic methyl 3α,7α-ditrifluoroacetoxy-Δ^{11}-cholenoate (Fig. 13). The enhanced m/e 340 ion in

the spectrum of the unknown compound over that in the spectrum of 3α,7α-di-trifluoroacetoxy-Δ^{11}-cholenoate strongly suggests the presence of additional ring C unsaturation, arising from the loss of the 7α-trifluoroacetoxy group. This favors the identification of the unknown compound as methyl 3α-trifluoroacetoxy-$\Delta^{8(14)}$,Δ^{11}-choledienoate. The remaining monotrifluoroacetoxy derivatives of the diunsaturated compounds (peaks 1 and 2) were eluted from the OV-210 column with retention times that indicated the retention of a 12α-trifluoroacetoxy group (29). This was further supported by the mass spectrum (Fig. 14), which indicated that the compound tended to lose the side chain, which is characteristic of 12-substituted bile acids. The absence of an ion at m/e 340 excluded ring C unsaturation and suggested the presence of a Δ^7-double bond. The spectra of both peak 1 and peak 2 showed close resemblance to that of methyl 3α,12α-ditrifluoroacetoxy-Δ^7-cholenoate (32,53) and the presence in the spectrum of peak 2 of an enhanced m/e 314, representing an RDA ion, suggests that these degradation products are the Δ^2- and Δ^3-isomers of methyl 12α-trifluoroacetoxy-Δ^7-cholenoate.

The presence of methyl 3α,12α-di-trifluoroacetoxy-$\Delta^{8(14)}$-cholenoate as the major breakdown product of methyl cholate tri-trifluoroacetate points out the preferential loss of the 7α-trifluoroacetoxy (or 7α-hydroxy) function over either the 12α- or the 3α-substituent. This is confirmed by the absence of 7α-substituted products among the monotrifluoroacetoxy-diunsaturated and di-trifluoroacetoxy-monounsaturated components. The relative order of the loss of the functional groups from the trifluoroacetoxy cholanoic acid methyl ester therefore may be summarized as follows: 7α > 12α > 3α. In contrast, the loss of an acetoxy group from the molecular ion of the acetoxy derivative occurs in the following order of decreasing preference: 12α > 7α > 3α (54).

A comparable order of degradation of saturated bile acids would be anticipated in the presence of hot (100 C) 5% HCl and methanol, which constitutes a recommended (55) method for the methylation of fecal bile acids. Likewise, the loss of TMS groups upon storage of the TMS ethers of bile acids or during their chromatography at high temperatures may result in similar degradation products (28,29), leading to a false indication of the natural occurrence of unsaturated bile acids. A systematic review of the routines commonly employed in the isolation and derivatization of bile acids shows that dehydration of the common bile acids may take place at any one step,

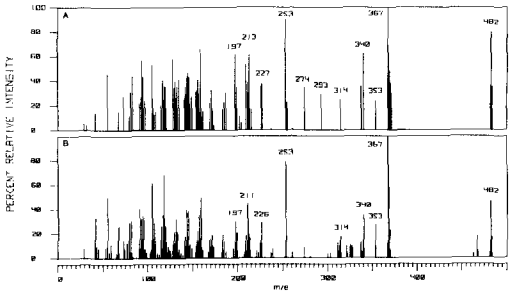

FIG. 13. GC-MS spectrum of a diunsaturated methyl 3α-trifluoroacetoxy-5β-cholanote (A) and of the closely similar spectrum of synthetic methyl 3α,7α-ditrifluoroacetoxy-Δ^{11}-5β-cholenoate (B). GC-MS conditions as in ref. 23.

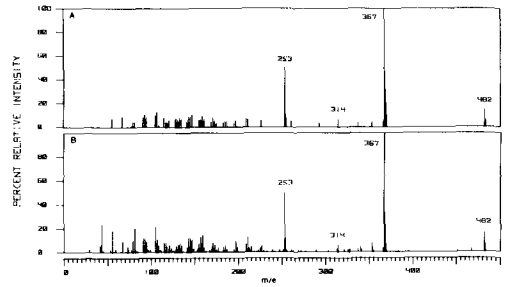

FIG. 14. GC-MS spectrum of a diunsaturated methyl 12α-trifluoroacetoxy-5β-cholanoate (A) and of a closely similar spectrum of synthetic methyl 3α, 12α-ditrifluoroacetoxy-Δ^7-5β-cholenoate (B). GC-MS conditions as in ref. 23.

but that it is most serious during methylation at elevated temperatures and during saponification of bile acid extracts containing sulfates (20,30). Diazomethylation (56), methylation with dimethoxypropane-HCl in the cold (34) as well as acetylation with acetic anhydride/pyridine (55) or acetic anhydride/perchloric acid (35) at moderate temperatures seem to be essentially free of dehydration products, but such sensitive methods as single-ion monitoring of the GLC profiles of the bile acid derivatives may still reveal minor amounts of unsaturated bile acids (22).

In the early work on bile acid GLC (56-59), methods were established for rapid and presumably artifact-free formation of volatile derivatives, and apparently satisfactory results were obtained. In several instances, where multiple peaks were obtained from a single compound, incomplete derivatization and/or hypothetical functional group interactions were proposed as an explanation (29,51). The advent of GC-MS methods has demonstrated that, in many instances, the extraneous peaks are dehydration

products (26,27,47). This study does not deny the natural occurrence and metabolic importance of unsaturated bile acids, but it suggests a need for great caution in the isolation and derivatization of bile acids intended for assay of unsaturated components.

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Conformational Analysis of Sterols: Comparison of X-Ray Crystallographic Observations with Data from Other Sources

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ABSTRACT

Crystallographic data on over 400 steroids collected in the *Atlas of Steroid Structure* provide information concerning preferred conformations, relative stabilities and substituent influence on the interactive potential of steroid hormones. Analysis of these data indicates that observed conformational details are intramolecularly controlled and that the influence of crystal packing forces is negligible. Crystallographic data on the orientation of the progesterone side chain contradict published force-field calculations. In 84 of 88 structures having a 20-one substituent, the C(16)-C(17)-C(20)-O(20) torsion angle is between 0° and -46°. The 4 torsion angles that lie outside this range do so because of a 16β-substituent and not because of crystal packing forces. Not one of the 88 structures is found to have a conformation in which the C(16)-C(17)-C(20)-O(20) torsion angle is within ±15° of the most commonly calculated minimum energy value. The narrow range of side chain conformations seen in very different crystalline environments in the 88 crystal structure determinations and the predictable substituent influence apparent in the data strongly suggest that crystallographically observed conformers seldom deviate from minimum energy positions, regardless of hypothetical broad energy minima, metastable states and small barriers to rotation. The 96 crystallographically independent determinations of the cholestane 17-side chain show that the chain has 4 principal conformations (A:B:C:D), occurring in the ratio 69:8:8:11. Although the fully extended side chain is clearly the energetically most favored one, in 16 observations of cholesterol itself only 6 are in the extended conformation. Some of the correlated conformational changes in the chains can be rationalized on the basis of model studies, but others apparently result from subtle intramolecular forces. The unsaturated B ring provides another element of flexibility in the structure of cholesterol. The 5-ene B ring is normally observed in an 8β,9α-half-chair conformation. However, in structures containing more than one molecule in the crystallographic asymmetric unit, at least one of the 2 molecules is found to differ significantly from this form. It may be that this inherent flexibility is responsible for the presence of conformationally distinct molecules in the same crystal. The intermolecular interaction observed in the crystal structure of cholesterol and its fatty acid derivatives illustrate the type of interaction between the steroid ring system and hydrocarbon chains that can be expected in membrane bilayers.

INTRODUCTION

Many biological functions have been shown to depend directly on the presence of a particular steroid. However, even in the most thoroughly studied systems, the nature of the molecular events involved in this structural-functional dependence is not fully understood. This highly sensitive correlation between steroid structure and observed function marks the steroid field as one of the most promising in which to pursue a molecular elucidation of structure-function relationships.

X-Ray crystal structure determinations provide the most precise and accurate details possible of molecular composition, configuration and conformation. Consequently, molecular structural data derived from X-ray studies are playing an ever-increasing role in developing understanding and control of biological processes.

Crystallographic data for 285 estranes, androstanes and pregnanes have been assembled and systematically presented in the first 2 volumes of the *Atlas of Steroid Structure* (1). In this paper, the data concerning the orientation of the 17β side chains of progesterone and cholesterol, the conformation of the 5-ene B ring of cholesterol and the crystal packing patterns observed in cholestanes are analyzed and compared with theoretical calculations when available.

DATA ANALYSIS

Progesterone Side Chain

The orientation of the progesterone side chain (Fig. 1) can be defined by the torsion angle C(16)-C(17)-C(20)-O(20) (2). If this angle is 0°, the O(20) carbonyl eclipses the C(16)-C(17) bond. If it is 120°, the carbonyl eclipses the C(13)-C(17) bond. The 88 pregnane structures having a 20-one substituent for which

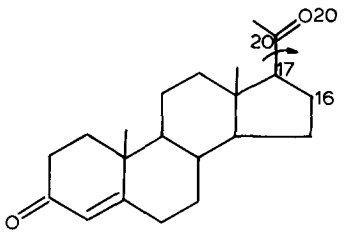

FIG. 1. *Progesterone structure with numbering of* the torsion angle that defines rotation about the C(17)-C(20) bond.

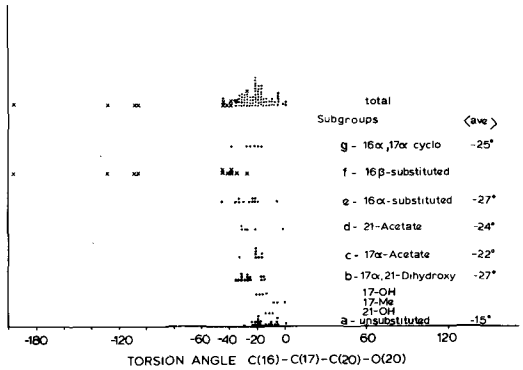

FIG. 2. The observed distribution of the torsion angle C(16)-C(17)-C(20)-O(20) in 88 structures having a 20-one substituent illustrates restricted rotation of the side chain about the C(17)-C(20) bond. The influence of various substitutions in subgroups a through g is also shown.

X-ray crystal structures have been reported provide an exceptionally large sample on which to base a reliable evaluation of the minimum energy position of the side chain and the influence of various substituents on the conformation. The C(16)-C(17)-C(20)-O(20) torsion angles for the 88 crystallographically distinct observations are shown in Figure 2. The distribution of subsets having (a_1) no additional substitution or unsaturation in the D-ring or side chain, (a_2) 21-hydroxy substitution, (a_3) 17α-methyl substitution, (a_4) 17α-hydroxy substitution, (b) 17α,21-dihydroxy substitution, (c) 17α-acetate substitution, (d) 21-acetate substitution, (e) 16α-substitution, (f) 16β-substitution, or (g) 16α,17α-cyclo substitution also are shown. Structures qualifying for inclusion in more than one of these groups have been included in the group in which the substituent apparently has the greatest influence on side chain orientation.

Cholesterol 17β-Side Chain

There are at least 96 crystallographically independent determinations of the conformation of the cholestane 17-side chain. (A third volume of the *Atlas of Steroid Structure* will include structures of cholanes, cholestanes, ergostanes and cardenolides.) The conformation of the side chain can be unambiguously defined by 6 torsion angles:

ω_1 = C(13)-C(17)-C(20)-C(22),
ω_2 = C(17)-C(20)-C(22)-C(23),
ω_3 = C(20)-C(22)-C(23)-C(24),
ω_4 = C(22)-C(23)-C(24)-C(25),
ω_5 = C(23)-C(24)-C(25)-C(26),
ω_6 = C(23)-C(24)-C(25)-C(27).

Although the terminal carbons C(26) and C(27) are chemically indistinguishable, they can be unambiguously labeled by adopting the following convention. Looking along the H(25)-C(25) bond, the clockwise sequence C(24), C(26), C(27) is imposed upon the atoms bound to C(25). Such a convention has been adopted in this analysis. When the C(25)-C(26) bond is *trans* to the C(23)-C(24) bond, the terminal

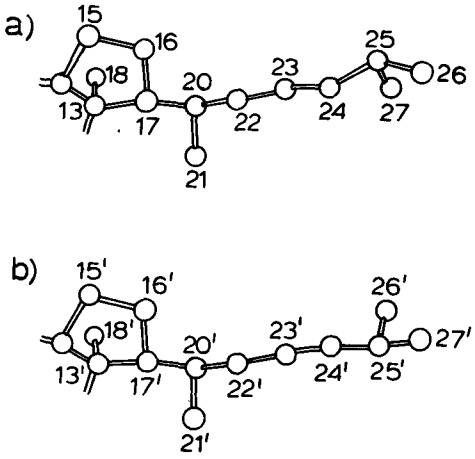

FIG. 3. Differences in conformation of the terminal methyls of the cholestane side chain in 5α-cholest-6-ene-3-one (10). *Trans,+gauche* (a) and *-gauche,trans* (b).

methyl conformation is said to be *trans,+gauche* (Fig. 3a). When the C(25)-C(27) bond is *trans* to the C(23)-C(24) bond, the conformation is *-gauche,trans* (Fig. 3b). The observed values of these 6 torsion angles are given in Table I. The data clearly define 4 primary conformers, the fully extended chain and 3 conformers (B, C, and D) distinguished by (+)synclinal conformations of ω_4, ω_3 and ω_2, respectively. The D conformers are further subdivided according to the value of ω_4.

Cholesterol B-Ring

The conformations of B-rings having a C(5)-C(6) double bond generally range about

TABLE I

Conformations of Cholestane Side Chains

References (structure)	ω_1	ω_2	ω_3	ω_4	ω_5	ω_6
			Conformer A			
3.*	-175	-168	-179	-176	-176	3
4.	178	-161	175	177	-172	15
5(a).	180	-164	174	-174	-171	33
6(a).	178	-159	176	-159	-170	34
7(a).	177	-169	148	175	-168	35
8.	178	-180	165	166	178	37
9(a).	180	-162	-178	-174	-170	37
10(a).	180	-166	178	178	-176	41
11(a).	-178	-178	178	167	-162	45
12(a).	176	-173	168	164	178	54
13(a).	175	-173	180	180	-172	56
11(b).	-178	-176	178	160	-174	56
14(a).	180	-173	-170	-166	166	57
15.	-179	-172	173	167	-177	57
16(a).	177	-178	174	177	-153	58
17.	-177	-164	-172	-173	180	59
18(a).	179	177	176	166	-172	59
19.	176	-172	178	165	179	59
11(c).	-178	-175	178	173	-170	59
20(a).	180	-174	176	174	-175	60
21.	-178	-156	-176	178	-175	61
12(b).	178	-172	171	172	-174	61
22.	-179	-155	-178	-179	-173	62
23.	177	-164	178	174	-169	62
24.	174	-177	172	177	-173	63
25.	-177	-153	-178	179	-173	63
26.	-180	-170	-178	175	-175	64
27.	-177	-158	-180	-180	-169	65
28.	-178	-173	168	169	-171	65
29.	177	-167	175	172	-171	66
11(d).	-179	-179	177	168	-170	66
30.	176	-165	177	178	-173	66
31(a).	177	-160	177	-174	-174	68
32.	179	157	180	-176	-169	69
33.	-178	179	175	-176	-165	71
18(b).	175	173	-178	175	-165	72
31(b).	-174	175	177	-174	-170	73
11(e).	177	-170	171	179	-166	75
11(f).	178	-169	175	167	-168	85
34.	178	-168	180	171	-154	85
35(a).	178	-176	-175	178	-150	89
36(a).	-175	-169	-179	160	-158	120
37(a).	-180	-173	176	176	-105	116
38.	175	-145	174	169	-80	155
39.	168	-178	-158	-162	-80	129
40.	180	-168	172	175	-78	157
41.	180	-173	171	-168	-74	165
42	-177	-173	162	-169	-73	-176
43.	-179	-165	175	-176	-72	164
44.	-178	-166	175	-172	-68	167
45.	180	-170	172	180	-67	168
46.	166	-168	166	-178	-67	172
47.	176	-151	-173	175	-67	-173
16(b).	177	-178	174	177	-66	129
5(b).	-177	-171	175	163	-64	168
48(a).	-172	-167	-174	177	-63	152
49.	-178	-164	-175	-170	-61	175
5(b).	-177	-170	-178	-175	-61	169
20(b).	-177	-160	-164	-166	-57	176
50.	176	-163	168	-174	-57	171
51.	173	-169	-179	178	-56	169
52.	178	-170	176	-174	-51	-176
53(a).	-178	-151	-177	-154	-49	-145
53(b).	-176	-173	177	-168	-49	172

TABLE I, continued

References (structure)	ω_1	ω_2	ω_3	ω_4	ω_5	ω_6
54.	177	-173	176	-160	-47	173
55.	178	-152	-179	-173	-39	174
9(b).	-173	-161	175	161	-39	164
35(b).	174	-171	171	166	-32	55
10(b).	-178	-164	-178	-175	-12	-176
			Conformer B			
56.	-170	-166	170	69	169	42
57.	180	-174	174	60	180	56
58(a).	-178	180	172	70	179	77
58(b).	-176	-166	-174	68	-170	80
58(c).	-174	-164	177	62	-179	80
58(d).	-175	-177	169	66	-179	85
59(a).	180	-160	180	109	-145	159
48(b).	-170	-154	-178	-40	-80	167
			Conformer C			
58(e).	174	180	59	168	-140	108
58(f).	174	-176	62	163	-136	110
58(g).	169	180	72	-176	-89	157
58(h).	174	-171	56	-173	-85	164
14.	176	175	85	180	-85	165
60.	175	178	56	176	-61	177
36(b).	-178	-168	72	-178	-54	-170
13(b).	180	176	81	177	-52	178
			Conformer D_1			
11(g).	174	63	175	-68	-64	176
11(h).	174	60	174	-67	-61	177
59(b).	173	66	-168	-71	-58	175
61.	179	73	-170	-99	-53	178
62.	-176	70	-166	-87	-39	-168
			Conformer D_2			
63.	177	62	158	-165	-172	47
37(b).	171	58	-180	173	-93	79
64.	174	60	-180	-170	-59	176
7(b).	-174	86	-170	-156	-3	171
			Conformer D_3			
65.	174	65	178	64	178	56
66.	172	57	176	61	-170	70

*Structures: 3. 4′,4′-dichloro-2β,3β-dihydrocyclobuta[2,3]-5α-cholestan-3′(4′*H*)-one; 4. cholesteryl octanoate; 5. cholesteryl dodecanoate; 6. cholesteryl nonanoate; 7. sodium cholesteryl sulfate dihydrate; 8. 5(10→1β*H*)*abeo*-10(19)-cholest-ene-3β,5α-diol 3-*p*-bromobenzoate; 9. cholesteryl laurate; 10. 5α-cholest-6-en-3-one; 11. anhydrous cholesterol; 12. cholesteryl acetate; 13. 14α-methyl-9β,19-cyclo-5α-cholestan-3β-yl acetate; 14. cholesteryl (17-bromo)heptadecanoate; 15. (3R)-5α-cholestan-4-one-3-spiro-2′-(1′,3′-oxathiolane); 16. 6β-chloroacetoxy-3,5α-cyclo-5-cholestane; 17. 25-hydroxyvitamin D_3 monohydrate; 18. 14α,15α-epoxy-5α-cholest-7-en-3β-yl *p*-bromobenzoate; 19. 5α-cholest-8(14)-ene-3β,15β-diol 3-*p*-bromobenzoate; 20. 4,4-dichloro-2a-*aza*-A-*homo*-coprostane; 21. 4-cholest-en-3-one; 22. 2α,3β-dichloro-5α-cholestane; 23. cholesteryl chloroformate; 24. 5-chloro-5α-cholestane; 25. 2α,3β-dibromo-5α-cholestane; 26. 2α-bromo-2β-methyl-5α-cholestan-3-one; 27. 4-cholest-en-6-one; 28. (E)-3α-acetoxy-5,10-*seco*-1(10)-cholesten-5-one; 29. 4β,5-epoxy-5β-cholestan-3-one; 30. 5α-cholest-2-ene; 31. (25R)-cholest-5-ene-3β,26-diol; 32. 1(10→16)*abeo*-5,7,9-cholesta-trien-3α-yl *p*-bromobenzoate; 33. 9β,10α-cholesta-5,7-diene-3β-ol ethanol (1:1); 34. 3′3′,-dichloro-2β,3β-dihydrocyclobuta[2,3]-5α-cholestan-4′(3′*H*)-one; 35. 2-*aza*-A-*homo*-5α-cholestan-1-one; 36. cholesteryl myristate; 37. 24-methylene-5-cholest-ene-3β,7β,19-triol; 38. 3β,16β,23(R),26-tetrahydroxy-5β-cholestane; 39. cholesteryl bromide; 40. 14α-methyl-5α-cholest-7-en-3β,15β-diol 3*p*-bromobenzoate; 41. aplysterol 3β-iodobenzoate; 42. 6β-Bromoacetoxy-3,5α-cyclo-5-cholestane; 43. 2β,3α-dichloro-5α-cholestane; 44. 4β,5β-epoxycholestan-6-one; 45. 6β-methyl-4-cholesten-

3-one; 46. 5α,14β-cholest-7-ene-3β,15β-diol di-*p*-bromobenzoate; 47. 3β-iodo-5-cholestene; 48. cholesteryl decanoate; 49. 5-vinyl-A-*nor*-5α-cholestan-3-one; 50. (25*S*)-3-oxo-4-cholesten-26-yl *p*-bromobenzoate; 51. 7α-bromo-3β-chloro-5-cholestene; 52. 3,5-dinitrobenzoate ester of toxisterol; 53. cholesteryl undecanoate; 54. cholesteryl chloride; 55. cholesteryl oleate; 56. 4,4-dichloro-2a-*aza*-A-*homo*-5α-cholestan-3-one; 57. 5-cholesten-3β-yl *p*-toluenesulfonate; 58. cholesterol monohydrate; 59. vitamin D_3; 60. (25*S*)-5-cholesten-3,25-26-triol; 61. (20*S*,22*R*)-2β,3β,14α,22,25-pentahydroxy-5β-cholest-7-en-6-one (ecdysone); 62. (20*R*,-22*R*)-2β,3β,14α,20,22,25-hexahydroxy-5β-cholest-7-en-6-one (20-hydroxy ecdysone); 63. 5-oxo-5,10-*seco*-1(10)-cholesten-3β-yl *p*-bromobenzoate; 64. cholesteryl dihydrocinnamate; 65. 6β-iodo-3β,5-epoxymethano-4-oxa-5β-cholestane; 66. 3α,5-dibromo-5β-cholestane.

the symmetric 8β,9α-half-chair conformation in which atoms C(10), C(5), C(6) and C(7) are coplanar because of the double bond and atoms C(8) and C(9) are displaced on the β- and α-sides of the plane, respectively (67). Rings having an ideal 8β,9α-half-chair conformation possess pseudorotation symmetry about an axis joining the midpoints of the C(5)-C(6) and the C(8)-C(9) bonds. If C(8) becomes coplanar with the C(10), C(5), C(6) and C(7) atoms, the B-ring has a 9α-sofa conformation and possesses a pseudomirror plane perpendicular to the ring and intersecting atoms C(6) and C(9). If C(9) rather than C(8) comes into the plane of C(10), C(5), C(6) and C(7), the pseudomirror plane passes through C(5) and C(8) and the conformation is an 8β-sofa.

The asymmetry parameters of nonideal systems measure the degree of departure from ideal symmetry (i.e., asymmetry). Related torsion angles are compared in a way that will result in a value of zero if the symmetry in question is present. Mirror-related torsion angles are inversely related (same magnitude, opposite sign) and such torsion angles are compared by addition. The 2-fold related torsion angles are directly related (same magnitude and sign) and are compared by subtraction. The root-mean-square synthesis of these individual discrepancies then yields a measure of the ring's deviation from ideal symmetry at the symmetry location in question. The 2 equations used to calculate the asymmetry parameters are:

$$\Delta C_s = \sqrt{\frac{\sum_{i=1}^{m}(\phi_i + \phi_i')^2}{m}} \qquad [I]$$

$$\Delta C_2 = \sqrt{\frac{\sum_{i=1}^{m}(\phi_i - \phi_i')^2}{m}} \qquad [II],$$

where m is the number of individual comparisons and ϕ_i and ϕ_i' are the symmetry related torsion angles. Equation I is used to calculate mirror plane asymmetry parameters (ΔC_s). Similarly, Equation II is used to calculate the 2-fold asymmetry parameters (ΔC_2) (67). The asymmetry parameter ΔC_2 (5-6) describes the deviation of a B-ring from the 8β,9α-half-chair conformation. A ΔC_2 (5-6) value of zero indicates a perfect half-chair conformation. The asymmetry parameter ΔC_s (6) describes the deviation of the ring from a perfect 9α-sofa conformation.

The calculated values of ΔC_2 (5-6) and ΔC_s (6) for all 49 5-cholest-ene steroids of Table I are given in Table II and plotted in Figure 4. Different symbols are used in the plot to distinguish between cholesterol and other cholestanes.

DISCUSSION

Progesterone Side Chain

In 84 of 88 steroids having the 20-one function (Fig. 2), the C(16)-C(17)-C(20)-O(20) torsion angle is between 0° and -46°. These observations are in agreement with the conformation predicted by Wellman and Djerassi (68) on the basis of optical rotatory dispersion and circular dichroism measurements. The 4 structures that lie outside this range do so because of a 16β-substituent and not because of crystal packing forces. Neither 17α-hydroxy, 17α-methyl, nor 21-hydroxy substitution alone has a significant influence on side-chain orientation. However, any one of 17α,21-dihydroxy substitution, 21-acetoxy or 16α-substitution shifts the side chain away from the normal position by an average of 10°.

Force-field calculations (69) have been published concerning 15 pregnanes with 20-one function. Energy profiles associated with side-chain rotation suggest (a) that the barrier to rotation about the C(17)-C(20) bond is never greater than 6 Kcal/mol, (b) that in many steroids (progesterone, 21-hydroxyprogesterone, 16α-methylprogesterone and 16β-methylcortexolone) the minimum energy conformation of the side chain is one in which the C(16)-

C(17)-C(20)-O(20) torsion angle is at or near -60° and (c) that in 16β-substituted structures there is a broad energy minimum extending between the C(16)-C(17)-C(20)-O(20) angles of -60° and -120°. Crystallographic data on 88 pregnane structures having a 20-one sbustituent contradict these conclusions. In Figure 5a, the energy profiles associated with side-chain rotation in progesterone and 21-hydroxy-progesterone are compared with the distribution of side-chain conformations observed in subset a. The observed population is shifted ca. 45° from the calculated minimum position. Surprisingly, the shift is toward the steep side of the well.

The distribution of the conformations of the side chains of the 16β-substituted structures suggests that a barrier to their interconversion lies in the region where C(16)-C(17)-C(20)-O(20) = -60°. Such a barrier is absent from the curves for the 16β-substituted cortexolone and progesterone (Fig. 5b).

The narrow range of side-chain conformations seen in very different crystalline environments in the 88 crystal structure determinations and the predictable substituent influence apparent in the data strongly suggest that crystallographically observed conformers seldom deviate from minimum energy positions, regardless of hypothetical broad energy minima, metastable states and small barriers to rotation. The presently used minimum energy calculation programs fail to represent intramolecular forces accurately and do not produce minimum energy conformations.

TABLE II

Conformations of B-Rings of 5-Cholest-enes As Described by Deviation from Ideal Forms, Ranked in Decreasing Order of ΔC_S (6)

ΔC_S (6)	ΔC_2 (5-6)	References (structure)
28.2	8.3	12(a)
27.3	7.7	31(a)
26.4	2.5	14(b)
25.4	2.7	14(a)
25.2	8.0	51
24.9	6.4	9(a)
23.2	1.1	11(c)
22.5	1.0	11(b)
22.3	1.7	64
22.1	2.3	41
22.0	2.6	57
21.8	5.7	58(c)
21.8	1.1	7(a)
21.3	2.3	53(a)
21.1	3.4	11(d)
20.8	2.2	23
20.8	3.4	6(a)
20.4	2.9	6(b)
20.3	2.9	11(a)
19.9	2.7	36(a)
19.8	5.5	37(b)
19.4	3.6	4
18.7	4.4	55
18.7	6.0	11(h)
18.4	2.0	48(a)
18.3	3.8	5(b)
18.1	4.6	7(b)
17.5	4.2	5(a)
17.4	7.7	37(a)
17.4	4.6	11(f)
17.3	5.7	11(g)
16.9	10.3	36(b)
16.5	6.6	11(e)
16.2	7.5	53(b)
16.1	6.7	60
15.6	6.6	47
15.6	11.6	54
15.3	4.7	39
15.0	6.2	9(b)
14.3	9.1	12(b)
12.8	14.7	31(b)
12.0	15.9	48(b)
11.3	6.9	58(h)
11.0	4.7	58(b)
9.9	12.9	58(a)
9.2	11.9	58(d)
7.8	15.6	58(e)
6.7	16.6	58(g)
6.4	18.2	58(f)

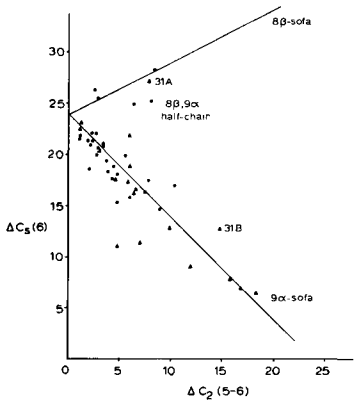

FIG. 4. Conformational variation in 5-ene steroids (Δ = 3-OH-5-ene-cholestanes, ● = other cholestanes).

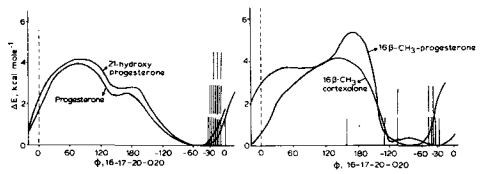

FIG. 5. (a) Energy profiles associated with side-chain rotation for progesterone and 21-hydroxy-progesterone compared to crystallographic observations. (b) Energy profiles associated with side-chain rotation for 16β-methylprogesterone and 16β-cortexolone compared to crystallographic observations.

Cholesterol Side Chain

The 6 principal conformations of the cholesterol side chains are illustrated in Figure 6. The relative occurrence of conformers A, B, C and D is 69:8:8:11. Failure to observe the -synclinal conformation at ω_2 and ω_3 can be rationalized on the basis of model studies. Within the conformational types, subgroups can be distinguished that illustrate correlations in the conformations of adjacent torsion angles. The torsion angles ω_4 and ω_5 are *+gauche,-trans* in conformers of types B and D_3, *trans, -gauche* in type C conformers and *-gauche,--gauche* in type D_1 conformers. These correlated differences can also be rationalized on the basis of model studies. The observed preference for the +synclinal conformation at ω_4 in conformer B and the -synclinal conformation at ω_5 in conformer C are not readily explained on the basis of model studies. Although the observation of a *-gauche* conformation of ω_4 in structure 48b demonstrates that the conformation is attainable, it should be noted that torsion angle ω_2 in this structure deviates significantly from 180°. The most unexpected result of this analysis concerns the distribution of observed conformation of the 5 terminal carbon atoms. Of the 2 most probable conformations *(trans,+gauche/-gauche,trans)*, one is preferred over the other by a ratio of 2:1 in the 69 structures with fully extended chains (conformer A).

There are 18 structures in Table I in which there are 2 molecules in the crystallographic asymmetric unit. In 17 of these structures at least one of the molecules has a fully extended side chain. In 3 cases (12,18,31), the side chains of both molecules have the *trans,+gauche* (ω_5,ω_6) conformation; in one case (53), both molecules have the *-gauche,trans* conformation; and in 7 cases, (5,6,9,10,16,20,35) *trans,-+gauche* and *-gauche,trans* conformers cocrystallize. Six more structures (7,13,14,36, 37,48) have one molecule with an extended side chain and a second molecule with a side chain in the B, C or D conformation.

All the evidence indicates that the fully extended side chain with a *trans,+gauche* (ω_5,ω_6) conformation is energetically favored. The repeated cocrystallization of *trans,+gauche* and *-gauche,trans* forms and this observed overall ratio of 2:1 suggest a small difference in their relative energies. The cocrystallization of cholestanes in which the side chain is in the extended form with those in conformation B, C, or D may indicate either comparable conformation energies of these forms under the condition of crystallization or the influence of

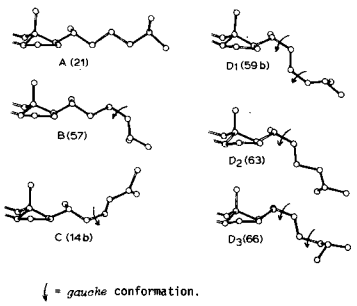

FIG. 6. Six different conformations of the cholesterol side chain.

crystal packing forces stabilizing a higher energy form. It is curious that in all but one case an extended conformer is present in the lattice. Apparently, crystal forces can rarely overcome the side-chain preference for the extended form. The only 2 structures having 8 molecules in the asymmetric unit are the anhydrous and the monohydrate forms of cholesterol. Six of the 8 chains in the anhydrous cholesterol crystals are in the typical extended, *trans,+gauche* conformation and the other 2 molecules have their side chain in the D_1 conformation. Surprisingly, none of the 8 molecules in cholesterol monohydrate has a side chain in the extended form. Four of the 8 chains in cholesterol monohydrate have one conformation (B), and there are 2 variants of conformer C present for a 4:2:2 ratio of conformational variation.

The 9 structures in the sample having side-chain substitutions (17,31a and b,37a and b, 38,41,50,60,61 and 62) have an A:B:C:D distribution of 8:0:1:3. Although substitutions do not seem to have a pronounced effect on the observed distribution of conformers, certain specific substitutions do have a characteristic effect. For example, the 22-hydroxy substituent in the 2 ecdysteroids (61 and 62) apparently is responsible for the adoption of a *+gauche* conformation of ω_2. It is also notable that although there is only one structure in which the C(25) hydrogen is observed to be *trans* to the C(23)-C(24) bond (48c), there are 5 conformers in which there is a near eclipsing of all 3 substituents on atoms C(24) and C(25), with the hydrogen substituents eclipsing methyl groups, i.e., ω_5 and ω_6 close to ±120°, suggesting that this conformation is preferred to one in which H(25) is *trans* to C(23)-C(24). It is stressed that the thermal motion at the end of the chain is large, particularly in structures having this unusual eclipsing, and that in several structures disorder is present.

Cholesterol B-Ring

The flexibility of the 5-ene B rings produces an appreciable twist about the length of the steroid. The 2 crystallographically distinct molecules in the double asymmetric unit of (25R)-cholest-5-ene-3,26-diol (31) provide examples of the extremes of this twist and its influence on the overall shape of the molecule (Fig. 7). More than half of the structures with 5-ene composition have multiple asymmetric units and in 8 of them (9,12,31,36,37,48,53 and 58) the B rings of the crystallographically distinct molecules exhibit differences similar to that shown in Figure 7.

Perhaps the most remarkable feature of Figure 4 is the wide range of flexibility of the B ring in structures with an unsubstituted 3-hydroxy group (Δ in Fig. 4). Cholesterol itself ranges in conformation from the perfect 8β,9α-half-chair conformation toward the 9α-sofa conformation. The B rings in the anhydrous form of cholesterol are in the more commonly observed conformation, but the B rings in the monohydrate crystals cluster near the midpoint between the 2 symmetric forms. As already noted, it is also in the monohydrate form that none of the side chains is fully extended. The flexibility of the rings as well as the side chain in cholesterol may contribute to the mobility of cholesterol in lipid bilayers.

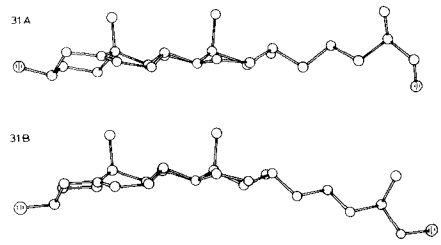

FIG. 7. Extremes of conformational variation in B-Rings of 5-ene steroids observed in (25*R*)-cholest-5-ene-3,26-diol (31) illustrate the impact on overall molecular conformation.

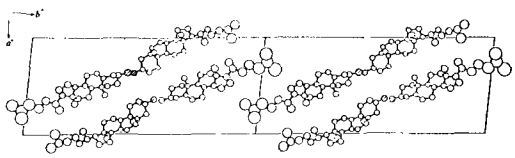

FIG. 8. Bilayer packing observed in an anhydrous cholesterol (11).

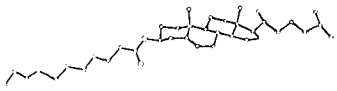

FIG. 9. Structure of cholesteryl undecanoate (53), illustrating fully extended fatty acid chain.

Cholestane Crystal Packing

The crystal structures of cholesterol and its fatty acid derivatives provide excellent models for intermolecular interactions in membrane bilayers. Cholesterol itself (11,58) forms bilayers of ca. 30 Å thickness with the polar regions at the outer surface (Fig. 8). The cholesterol side chains are interdigitated in the anhydrous form but not in the monohydrate. Craven and coworkers have reported an elegant series of crystallographic studies of fatty acid derivatives that illustrate a variety of types of interaction between the steroid ring system and the hydrocarbon chains. In most cases, the fatty acid chains are fully extended, as illustrated in Figure 9. In the myristate (36) and heptadecanoate (14), the steroid rings are packed adjacent to one another, the fatty acid chains are packed together and the cholesterol side chains are at the surfaces of bilayers, where thermal motion is highest (Fig. 10a). In the oleate (55) and octanoate (4) structures, the steroids are again packed adjacent to one another, but the fatty acid chains and the cholesterol side chains are packed together (Fig. 10b). In the laurate (5 and 9), decanoate (48), undecanoate (53) and nonanoate (6) derivatives, the fatty acid chains pack with the cholesterol tetracyclic systems and not with themselves (Fig. 10c). Craven points out that arrangements of this last type are presumed to exist when cholesterol is incorporated within biological membranes.

Other Correlations

In addition to providing a wealth of information on the conformation of the cholesterol B ring and side chain and illuminating the probable nature of interactions of cholesterol in biological membranes, the individual X-ray crystal structures have also answered a variety of biochemical and biophysical questions. The observed conformations of the lactone rings in 4,4-dichloro-2a-*aza*-A-*homo*-coprostane (20) and 4,4-dichloro-2a-*aza*-A-*homo*-5α-cholestan-3-one (56) were found to be consistent with ORD and CD spectra. A number of structures, analyzed as part of a study of mechanisms of sterol biosynthesis (19,40,41 and 50), resolved questions of connectivity and absolute configuration. A comparison of the A-ring conformation in 3-keto-5α-cholestanes provided quantitative evidence from solid state studies

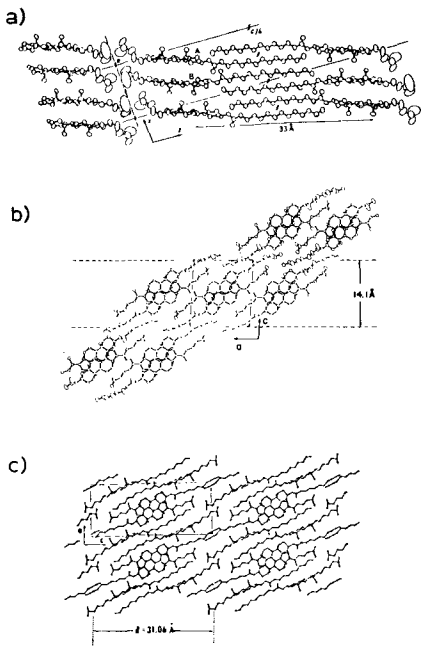

FIG. 10. Variety of packing types and steroid fatty acid interactions observed in crystals of (a) cholesteryl myristate (from 36), (b) cholesteryl octanoate (from 4), and cholesteryl undecanoate (from 53).

that the rate of condensation of these compounds with benzylaldehyde is governed by conformational transmission, as shown by the chemical studies of Barton et al. (70).

Vitamin D_3 crystallizes with 2 molecules in the asymmetric unit, and 2 different chair forms of the A ring are observed. These observations confirmed the 2 forms proposed to explain solution spectral data (71). Further, based on the activity and conformational stability of A-ring-substituted analogs of vitamin D_3, it has been proposed that one of the A ring conformations is essential to hormonal function. The cocrystallization of active and inactive conformers that are known to be in equilibrium in solution and presumed to be of approximately equal energy underscores the potential significance of the conformational differences observed in the solid state. The numerous cases (Table II) in which the 5-ene B rings of 2 molecules in the same crystal differ to the degree illustrated in Figure 7 suggest that this conformational difference may also have a biological significance.

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Determination of Molecular Formula and Stereoconfiguration of Unique Steroids by X-Ray Diffraction Analysis

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ABSTRACT

X-Ray diffraction analyses can determine chemical composition, molecular formula and stereoconfiguration and can provide geometric parameters with uncertainties for bond distances of the order of 0.01 Å and for bond angles of the order of 1°. These analyses are especially useful when the substance analyzed is present only in very small quantities and is of uncertain composition or molecular formula. Only one suitable crystal is required for the analysis to proceed. There are many instances in which such circumstances have prevailed in the diffraction analysis of steroids. Among the examples given here is a new type of natural plant hormone with profound growth-stimulating capabilities when applied in nanogram quantities per plant. The structure determination of this substance has greatly facilitated attempts to synthesize it and thereby to derive sufficient quantities for widespread testing.

INTRODUCTION

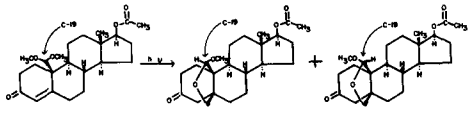
Single crystal X-ray diffraction analysis has provided the 3-dimensional arrangements of atoms in numerous steroidal substances. In many cases, the stereoconfiguration established by chemists by chemical and physicochemical means were merely confirmed by the X-ray structure analysis. Even then the X-ray analysis provided a quantitative insight into the structural characteristics of the analyzed substances, such as bond lengths, bond angles and conformational features, with the high accuracy that is presently found only among diffraction techniques. Such quantitative geometric details not only are of inherent interest, but when used for comparisons among closely related compounds they can also facilitate the understanding of varying physiological activities and suggest structural alterations to achieve desired results.

The use of diffraction analysis is particularly appropriate when there are uncertainties concerning the chemical composition, the structural formula or the stereoconfiguration, or when the quantities available are very small. The only requirement is the availability of a single crystal with dimensions ranging from about 0.1 to 0.5 mm. The steroidal structures to be described in this article are examples of these special circumstances. A particularly interesting example concerning the frog toxin, batrachotoxin, a steroidal alkaloid having profound neurological effects, has been described in an earlier article concerning the investigation of steroidal structures by diffraction analysis (1).

The method usually applied to the analysis of diffraction data for steroidal structures is the direct method of phase determination described in some detail in the earlier article (1). Steroids normally crystallize in noncentrosymmetric space groups, and because they usually are composed of nonhydrogen atoms that differ little in atomic number, they are among the more difficult structures to analyze. It should be noted, however, that the direct method for structure analysis proceeds without the necessity of introducing heavy atoms into the structure.

APPLICATIONS TO STEROIDS

There are many examples in which structure determination by X-ray analysis can play a key role, not only because the amounts of material available are very small, but also because unusual or extensive changes have occurred in the course of a reaction. Ultraviolet (UV) irradiation can generate unusual and, at times, extensive chemical changes in a substance. An example of an unusual change resulted from the UV-irradiation of 19,19-dimethoxy-17β-acetoxy-4-androsten-3-one. A major and a minor product were obtained which were thought to be epimers differing only in the configuration at C(19). The reaction is shown as follows:



(I) (II)

SCHEME I

An X-ray structure analysis was performed (2) to determine the stereoconfiguration of the major photoproduct, a substance that crystallized in space group $P2_1$. The result is illustrated in the stereodiagram (Fig. 1). It shows that

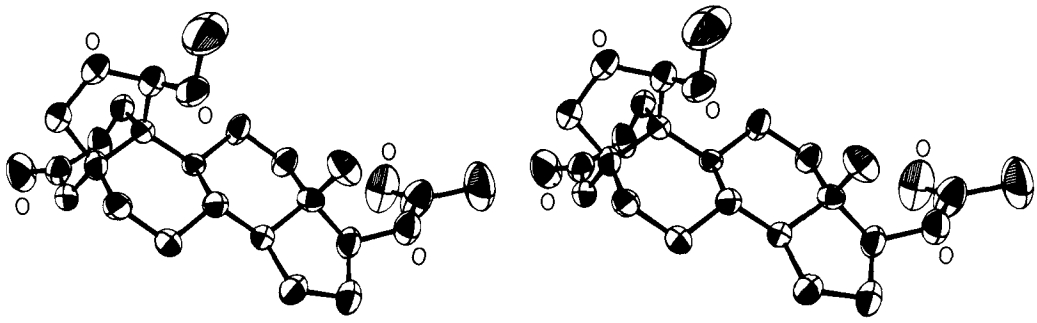


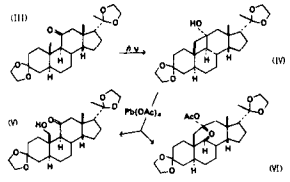
FIG. 1. A stereodiagram of the major photoproduct I (see Scheme I).

in the major product, the methoxy group attached to C(19) points toward C(18), as indicated in photoproduct I in Scheme I. It is also seen that an additional ring containing an oxygen atom is formed. The A/B ring junction is *cis*, whereas the B/C and C/D ring junctions are both *trans*. The addition of the 5-membered ring between C(5) and C(10) flattens rings A and B near their junction and the torsional angles in this region are 5-10° smaller than normal. The methyl group in the methoxy moiety on C(19) is *trans* with respect to C(10) with a torsion angle about the C(19)-O(3) bond of +177°.

Another investigation that originated in the use of UV-irradiation to generate chemical and structural changes concerned a derivative of pregnane. The irradiation of various derivatives of pregnane can induce cyclization between C(11) and the methyl group at C(19), forming a 4-membered ring (3). The reaction proceeds from structure III e.g., to the cycloproduct IV (Scheme II). Fragmentation of (IV) by $Pb(OAc)_4$ yields VI as the main product, rather than the expected product V.

As a consequence of the ring closure between C(11) and C(19) and the introduction of an oxygen atom between C(9) and C(11), the steroid nucleus apparently would be forced to undergo marked conformational changes. An X-ray structural investigation was undertaken to examine this matter in detail and also to confirm the formula and stereoconfiguration (4). The result of this investigation is shown in the stereodiagram in Figure 2. Some of the structural features of VI that accommodate the oxygen bridge formed between C(9) and C(11) and the ring closure by the C(11)-C(19) bond are seen in the values of some angles. For example, C(2)C(3)C(4) = 118°, C(10)C(5)C(6) = 120° and C(11)C(12)C(13) = 122° in rings A, B and C, respectively, which are considerably larger than the values usually found for tetrahedral carbon atoms.

Rings A and B are in the chair conformation and have a *trans*-junction, the 7-membered ring C is in the boat conformation and the 5-membered rings D, E and F are close to the half-chair form. Rings C and D have a *cis*-junction.



SCHEME II

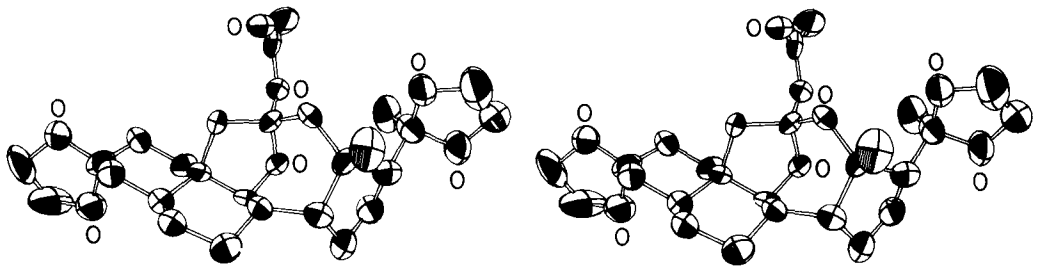
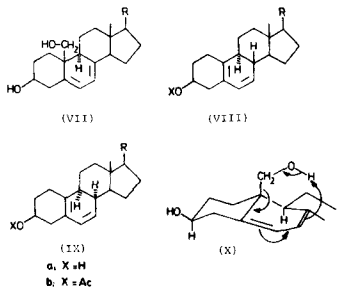


FIG. 2. A stereodiagram of a product derived by photocyclization of a pregnane derivative, VI (see Scheme II).

Ring G has an envelope form with C(9) out of the plane formed by the other 4 atoms.

In another UV-irradiation experiment (5) on 19-hydroxy-7-dehydrocholesterol (Structure VII, Scheme III), it was found that a loss of the functionality at C(10) occurs, yielding products VIIIa and IXa. Product VIIIa is the main photoproduct, whereas the diene IXa is obtained from VIIIa in a secondary photochemical reaction.



SCHEME III

The development of a possible mechanism for the formation of (VIIIa) and (IXa) is facilitated by knowledge of the stereochemistry of the B/C ring juncture. A likely model for this stereochemistry leads to the prediction of a positive Cotton effect, whereas a negative curve was observed. Therefore, an X-ray structural investigation was undertaken (5) and the results are shown in the stereodiagram in Figure 3. X-Ray analysis showed that the unsaturation in ring B caused distortions in the conformations of the 6-membered rings. Ring A has an envelope conformation and ring B has a half-boat conformation. Ring C is in the normal chair conformation, but some torsion angles lie outside the usual range of 55-60° for the chair conformation. Additional derivatives were prepared by deuteration and acetylation (VIIIb, Scheme III). From the X-ray analysis and configurational considerations, a mechanism for the loss of the C(10) functionality was proposed.

Of particular interest is some recent work on natural plant growth regulators. There is a worldwide need to produce both energy and food efficiently and safely. Recent advances in the use of plants for energy sources, such as the production of alcohols and crude oil, have greatly intensified the need to achieve reliably high yields of high-quality crops on a continuing basis, even in the face of adverse growing conditions. Since the dangers of nonbiodegradable pesticides have become well known, research on plant growth control has centered on naturally occurring substances produced by the plants themselves. These plant hormones are organic compounds other than nutrients, which, in low concentrations, affect the physiological processes of plants. The effective use of hormones to improve plant growth can greatly increase the production of biomass and even of plants that contain oil (6).

Many research projects at present concern the isolation of hormones from plants. Collecting, isolating and purifying these materials is an expensive, time-consuming process, and most efforts result in extremely small amounts of pure hormones, even when the original sample consists of several kilograms of raw material. They must then be chemically synthesized and manufactured for large-scale practical applications. Evidently, in order to synthesize a molecule, its structure must be known. Brassinolide (7-9) is a very potent plant growth promoter, active in amounts as low as 1-10 ng/per plant. Its isolation and purification has been of considerable interest since it has been shown to cause cell enlargement and to increase cell divisions in plants such as beans, corn and other vegetable crops. It also has shown potential for use in many other agricultural crops and in certain plants grown in arid regions that yield oil and other energy-related materials. However, the yield is so low (1 part in 10^5 parts of the original sample) that it would be impossible to consider large-scale testing unless the substance could be synthesized. A few crystals of brassinolide were obtained and, although they were of poor quality, it was possible to gather the necessary data for an X-ray structural investigation.

The structure of brassinolide has unusual features that would be quite difficult to determine by the usual chemical and physicochemical methods. The results of the X-ray analysis (9) shown in the stereodiagram in Figure 4 indicate that the 2,3-*cis*-glycol is α-oriented, all the ring junctions are *trans* and the B ring is

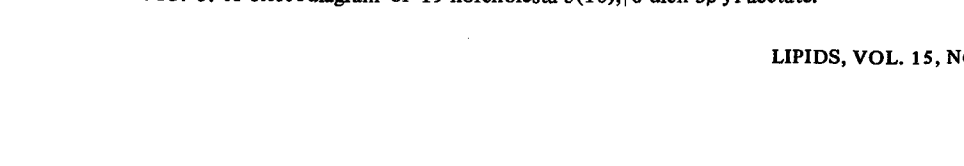


FIG. 3. A stereodiagram of 19-norcholesta-5(10),|6-dien-3β-yl acetate.

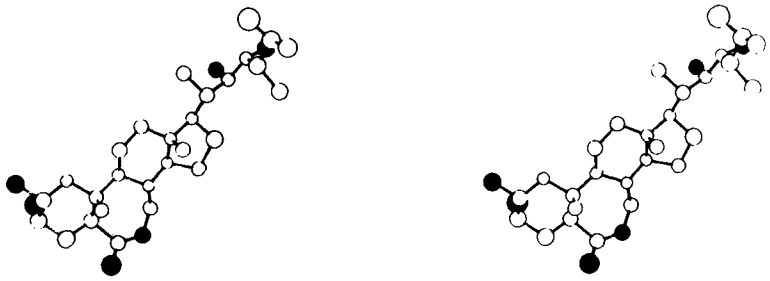
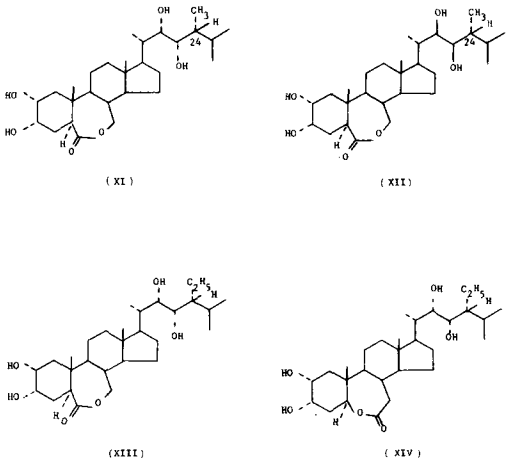


FIG. 4. A stereodiagram of brassinolide. The darkened circles represent oxygen atoms. All stereodiagrams in this manuscript were drawn from experimentally determined coordinates with the aid of the ORTEP computing program (10).

a lactone, an unprecedented feature in natural steroids. Only the relative configuration was determined in the X-ray analysis, but the enantiomer chosen for Figure 4 depicts the normal absolute configuration found in natural steroids.

Ever since brassinolide was isolated and characterized, attempts have been underway to synthesize not only brassinolide but also analogous compounds that are easier to prepare in large quantities and still show adequate plant-growth regulating properties. Four such materials, Structures XI and XII (Scheme IV) (11) and Structures XIII and XIV (J.L. Flippen-Anderson and R.D. Gilardi, personal communication), were investigated by X-ray structural analyses in order to establish the stereochemistry at C(22), C(23) and C(24). The X-ray analyses showed that the steroid nucleus of Structures XI and XII and brassinolide are identical but that brassinolide differs from XI at C(22), C(23) and C(24), whereas it differs from XII only in the interchange of the methyl group and the hydrogen atom at C(24). The biological activities of XI and XII are ca. 0.1 of the activity of brassinolide. Compounds XIII and XIV differ from XI in that C(24) is substituted with an ethyl rather than a methyl moiety and that XIV, in addition, has a different lactone ring. The X-ray studies confirmed the configurations of XIII and XIV. The biological activities of XIII and XIV are similar to those of XI and XII or ca. 0.1 of the activity of brassinolide.



SCHEME IV

CONCLUDING REMARKS

Evidently, there are many instances in which X-ray structure analysis has played an indispensable role in facilitating the investigation of steroidal substances. These include the identification of the chemical formula and the determination of stereoconfiguration. X-Ray analysis has facilitated chemical synthesis as well as the understanding of physical and chemical properties and reaction mechanisms.

There are close interrelationships between structure analyses and the calculations of theoretical chemistry. At present, increased efforts are being made to develop these interrelationships between structure analyses and the calculations of theoretical chemistry. At present, increased efforts are being made to develop these interrelationships by associating structure investigations with theoretical calculations. The objective is to obtain deeper insight into conformational characteristics, reactivities, bonding and intermolecular interactions. Future research on steroidal molecules should benefit from these developments.

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Fatty Acid Composition of Symbiotic Zooxanthellae in Relation to Their Hosts

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ABSTRACT

Gymnodinoid dinoflagellate symbionts, commonly referred to as zooxanthellae, are widely distributed among marine invertebrates. It has been assumed that they represent only one species, *Gymnodinium microadriaticum*. The fatty acid composition of total lipids and galactolipids of zooxanthellae isolated from 8 species of corals, 3 species of clams and a foraminiferan have been analyzed and found to vary according to the host. For example, the content of eicosapentaenoic acid in clam zooxanthellae monogalactosyldiacylglycerol was less than 2%, whereas in the same lipid from coral zooxanthellae, the content ranged from 9 to 22%. Corresponding values for the acid in digalactosyldiacylglycerol were 1-8% from clam zooxanthellae and 23-40% from coral zooxanthellae. Coral zooxanthellae monogalactosyldiacylglycerol contain higher levels of octadecatetraenoic acid than are found in digalactosyldiacylglycerol, whereas the reverse is true in clam zooxanthellae. The fatty acid composition of the lipids of an axenic culture of zooxanthellae isolated from the clam *Tridacna maxima* are similar to those of cells freshly isolated from the host. The results suggest either that the host is capable of affecting the fatty acid metabolism of the symbiont or that different strains of zooxanthellae occur in corals and clams.

INTRODUCTION

Dinoflagellate endosymbionts have been detected in a large variety of marine invertebrates. Of these, the most widespread are the gymnodinoid dinoflagellates commonly known as zooxanthellae, which occur in marine invertebrates ranging from the Protozoa to the Mollusca (1). These zooxanthellae have been regarded by Taylor as consisting of only one species, *Gymnodinium microadriaticum* (1). Recently, however, this view has been questioned by Schoenberg and Trench (2-4), who have detected biochemical, morphological and ultrastructural differences in zooxanthellae isolated from different hosts, and have shown that the capacity of algae isolated from one host to infect another can differ widely.

The lipids of dinoflagellates are characterized by high concentrations of polyunsaturated fatty acids (5,6) and in particular of octadecapentaenoic acid, which has so far been reported to occur only in the Dinophyceae (6). It is present in the lipids of zooxanthellae isolated from the clam *Tridacna maxima* and is concentrated in the galactolipids, which are the major lipid components of the chloroplast membranes (7). This report details the fatty acid composition of total lipid and galactolipids of zooxanthellae isolated from a variety of hosts in the tropical waters of the Great Barrier Reef, Australia. It shows that octadecapentaenoic acid is always present and that the fatty acid composition of the symbiont varies in different hosts.

METHODS

Corals and clams were collected in the vicinity of Great Palm Island (lat. 18° 45′S; long. 146° 40′E) and Lizard Island (lat. 14° 40′S; long. 145° 28′E) and stored in tanks of running sea water. Zooxanthellae were isolated as previously described (7,8). Lipids were extracted from duplicate or triplicate samples of isolated zooxanthellae, intact foraminifera and whole coral heads, with chloroform/methanol (2:1) in the presence of butylated hydroxytoluene as antioxidant. The extracts were shaken with 0.2 vol of 0.73% NaCl solution and the chloroform layer dried over Na_2SO_4, and concentrated under vacuum. All samples were stored in chloroform at -20 C in the presence of butylated hydroxytoluene. Analyses of galactolipids and fatty acids were carried out as previously described (7) except that quantitative analyses of fatty acids by gas liquid chromatography (GLC) were carried out on the SP-222-PS column at 175 C. Pure galactolipids for fatty acid analyses were isolated by preparative thin layer chromatography (TLC).

Axenic cultures of dinoflagellates were grown using the conditions described by Deane and O'Brien (9).

RESULTS

The fatty acid compositions of the total lipids of zooxanthellae isolated from 8 species of corals (representing 3 families), 3 species of clams (2 families) and that of a foraminiferan are shown in Table I. Octadecapentaenoic acid (18:5ω3) is present in all samples in amounts ranging up to 12% of the total fatty acids. Hexadecanoic acid (16:0) is a major component in every case, its content ranging

from 14 to 23%. γ-Linolenic acid (18:3ω6) previously reported as a component of clam zooxanthellae lipids (7) is also generally present in amounts much larger than α-linolenic acid (18:3ω3) although α-linolenic acid is the isomer which is normally found in plants. The identity of 18:3ω6 was confirmed by argentation chromatography of the methyl ester which was eluted in the trienoic acid fraction, and by mass spectrometry (MS). There are, however, marked differences in the levels of some polyunsaturated fatty acids of zooxanthellae from different hosts. Thus, octadecatetraenoic acid (18:4) constitutes 9-18% of the fatty acids of coral zooxanthellae, but over 30% of the fatty acids of clam zooxanthellae. Concomitantly, clam zooxanthellae fatty acids contain only small amounts of eicosapentaenoic acid (20:5) whereas this acid is a major component of coral zooxanthellae lipids. The high level of eicosatetraenoic acid (20:4) in the foraminiferan lipids probably reflects the fact that the total organism was extracted, rather than isolated zooxanthellae. In a similar fashion, the level of 20:4 in a total extract of *Acropora millepora* (polyp + zooxanthellae) was higher than that of the isolated zooxanthellae. Fatty acid analyses of the total lipids of gonads and adductor muscle of the clam *T. maxima* did not reveal the presence of any 18:5, suggesting that this acid is confined to the zooxanthellae. However, it was found that of the 2 isomers of 18:3, the content of 18:3ω3 was higher in both organs (2.6% in gonads, 2.4% in muscle) than that of 18:3ω6 (1.3% in gonads, 1.2% in muscle). This is the reverse of the situation in the symbiont.

The possibility that contaminating animal tissue might be affecting the analyses of zooxanthellae lipids cannot be excluded. This is particularly true of coral zooxanthellae where the presence of engulfing gastrodermal cells occasionally could be detected in the zooxanthellae preparation (8). Therefore, analyses were made of the fatty acid composition of monogalactosyldiacylglycerol (MGG) and digalactosyldiacylglycerol (DGG), which are the major lipid components of chloroplast membranes (10) and therefore reflect only the composition of the symbiont. The molar ratio of MGG:DGG, averaged over all the samples examined, was 1.03 ± 0.14 in coral zooxanthellae and 1.17 ± 0.14 in clam zooxanthellae. These differences are not considered significant.

The fatty acid composition of MGG and DGG from coral zooxanthellae is shown in Table II. Hexadecanoic acid (16:0), a major component of the total lipid fraction, is only a minor component of the galactolipid fatty acids, and there is also a decrease of docosahexaenoic acid (22:6) in galactolipids as compared to total lipids. The major fatty acid components of the galactolipids are 18:3ω6, 18:4, 18:5 and 20:5. Of these, 18:4 and 18:5 are always present at higher levels in MGG than in DGG, whereas the reverse is true for 20:5.

There are major differences in the galactolipid fatty acid composition of clam zooxanthellae and those of coral zooxanthellae. The major fatty acid in clam zooxanthellae is 18:4 which in *Hippopus hippopus* zooxanthellae accounts for almost 70% of the DGG fatty acid (Table III). Clam zooxanthellae DGG contains more 18:4 than does MGG, whereas the reverse is true in coral zooxanthellae. Clam zooxanthellae galactolipids have substantial amounts of 18:5 but the major variation between clam and coral zooxanthellae galactolipid fatty acid is in the level of 20:5. In coral zooxanthellae MGG, the content of this acid ranges from 9 to 22%, whereas in clam zooxanthellae MGG it is less than 2%. The contrast is more striking if one compares the level of 20:5 in clam zooxanthellae MGG with that of the *Acropora* zooxanthellae MGG where the content of this acid is over 18%. The lowest value in the level of 20:5 in coral zooxanthellae MGG occurs in the symbiont isolated from *P. damicornis*– a different family from the *Acropora*, and one which has shown other variations in fatty acid composition from the *Acropora* zooxanthellae. The content of 20:5 in clam zooxanthellae DGG is from 2 to 8%, compared to 23-40% in coral zooxanthellae DGG.

The fatty acid composition of the galactolipids of the zooxanthellae from the foraminiferan *Marginopora* are similar to those from clam zooxanthellae, having a high content of 18:4 and a low content of 20:5 (Table III). There is only a small amount of 20:4 in *Marginopora* zooxanthellae galactolipids, compared to 21% in the total extract confirming that this acid is concentrated in the host tissue lipids.

Finally, Table IV shows the fatty acid analysis of total lipids and galactolipids of axenically grown cultures of zooxanthellae isolated from the clam *T. maxima*, and compares them to the lipids of *Amphidinium klebsii*, another dinoflagellate capable of symbiotic association, and those of *Amphidinium carterae*, a nonsymbiotic dinoflagellate. The axenic zooxanthellae from *T. maxima* contain substantial levels of 18:5 and 18:3ω6 but little 20:5 and they are devoid of 20:4, 22:4 and 22:5. There also is a marked increase in the levels of 16-carbon fatty acids, especially 16:1 and 16:3, in the axenic, compared to the symbiotic cells. Nevertheless, their fatty acid

TABLE I

Major Fatty Acids of Zooxanthellae Total Lipids

Host	Corals								Clams			Foraminiferan
	Acropora divaricata	*Acropora formosa*	*Acropora hyercinthus*	*Acropora millepora*	*Acropora nasuta*	*Leptastrea pruinosa*	*Montastrea curta*	*Pocillopora damicornis*	*Hippopus hippopus*	*Tridacna crocea*	*Tridacna maxima*	*Marginopora vertebralis*
Acid												
14:0	4.3	3.3	5.4	3.5	3.7	3.4	8.6	4.8	5.0	5.4	4.1	6.5[a]
16:0	18.8	19.0	18.2	23.1	16.6	18.1	21.6	20.0	14.5	17.7	14.6	18.5
16:1	2.0	3.9	3.4	2.0	2.9	3.9	2.9	2.8	1.7	3.4	1.6	1.0
18:0	4.4	4.4	6.4	6.9	4.7	7.1	5.2	7.2	2.3	1.4	1.6	11.7
18:1	2.1	3.3	3.2	3.4	1.9	3.7	4.1	2.8	4.3	3.4	3.0	3.5
18:2ω6	1.0	0.5	1.4	0.9	0.8	0.9	1.0	0.3	0.8	1.9	1.1	0.2
18:3ω6	14.5	9.2	7.4	6.2	8.1	8.3	8.3	2.8	1.4	7.7	12.3	0.2
18:3ω3	0.2	0.4	1.0	0.9	0.4	+[b]	+	0.9	2.4	0.2	1.2	1.2
18:4ω3	10.4	15.8	9.2	10.1	12.7	9.2	9.3	18.1	37.5	31.6	33.5	14.1
18:5ω3	6.7	4.3	4.8	4.6	7.6	5.3	5.9	1.8	12.8	12.5	10.4	3.2
20:4ω6	2.4	2.7	4.6	2.8	2.3	7.8	5.7	6.3	+	+	+	21.2
20:5ω3	20.9	17.4	17.3	20.0	22.8	16.1	15.2	9.0	0.4	1.8	3.3	1.4
22:4ω6	1.2	1.1	2.7	1.9	1.3	2.6	1.5	3.9	+	+	+	+
22:5ω3	0.4	0.2	1.2	1.8	0.8	0.2	+	+	+	+	+	+
22:6ω3	7.5	7.9	7.1	7.9	8.4	6.4	7.1	16.0	15.2	11.2	11.5	14.4

[a]The values represent analysis of the total lipid of both host and symbiont as it was not practical to isolate pure zooxanthellae from this host.

[b]A + sign indicates that the acid was detected but comprised less than 0.2% of the total fatty acids.

TABLE II

Major Fatty Acids of Coral Zooxanthellae Galactolipids

Host	*Acropora divaricata*		*Acropora formosa*		*Acropora hyercinthus*		*Acropora millepora*		*Acropora nasuta*		*Leptastrea pruinosa*		*Pocillopora damicornis*	
	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG
Acid														
14:0	1.0	10.8	2.7	1.7	2.4	4.9	2.1	2.0	0.3	1.2	4.4	12.6	1.6	2.4
16:0	2.7	2.2	2.3	1.1	3.3	2.3	3.6	2.8	2.4	1.7	1.3	2.0	2.1	3.9
16:1	1.2	0.8	2.2	0.9	1.5	1.2	2.1	1.2	1.4	0.9	2.6	1.4	1.1	1.3
18:0	0.5	0.5	0.3	0.3	0.3	0.3	2.6	1.3	0.5	0.4	+[a]	0.4	0.2	0.4
18:1	0.8	0.8	1.4	1.3	1.1	1.2	2.5	1.8	1.0	1.2	1.4	1.2	2.1	1.3
18:2ω6	1.0	0.6	0.5	0.3	0.7	0.6	2.0	0.4	0.7	0.5	0.7	0.5	0.6	+
18:3ω6	26.0	17.0	12.7	10.3	15.1	11.3	7.1	8.6	14.9	12.4	13.0	10.4	1.1	1.5
18:3ω3	-	+	-	+	-	0.7	2.0	+	+	0.2	-	-	+	+
18:4ω3	23.4	18.6	39.3	31.9	32.0	22.7	27.5	24.7	29.6	24.3	25.8	21.9	66.8	55.7
18:5ω3	20.8	10.9	14.9	8.8	23.9	13.4	18.2	12.2	24.0	13.5	22.7	10.9	10.8	6.1
20:4ω6	+	0.3	-	+	+	+	2.0	0.6	-	-	+	+	-	+
20:5ω3	21.2	35.0	19.2	34.7	18.9	36.6	19.9	36.1	22.9	40.5	22.3	32.6	9.0	22.9
22:4ω6	-	+	-	1.7	-	0.6	-	0.4	-	0.2	-	+	-	+
22:5ω3	-	+	-	0.3	-	0.7	-	1.0	-	0.4	-	0.3	-	-
22:6ω3	0.8	1.8	1.9	4.2	+	2.8	2.5	2.6	1.2	2.4	0.4	0.9	-	1.8

[a]A + sign indicates that the acid was detected but comprised less than 0.2% of the total fatty acids.

TABLE III

Major Fatty Acids of Clam and Foraminiferan Zooxanthellae Galactolipids

Host	Clams						Foraminiferan	
	Hippopus hippopus		*Tridacna crocea*		*Tridacna maxima*		*Marginopora vertebralis*	
Host	MGG	DGG	MGG	DGG	MGG	DGG	MGG	DGG
Acid								
14:0	1.8	0.8	1.2	1.2	0.7	1.0	1.7	16.1
16:0	1.7	1.4	1.6	1.8	1.1	1.4	2.5	5.8
16:1	+[a]	0.2	1.1	1.0	0.4	0.3	0.4	0.3
18:0	0.2	+	+	+	+	+	0.3	1.8
18:1	1.1	0.8	0.7	0.7	0.6	0.8	1.0	0.7
18:2ω6	0.6	0.8	0.9	0.8	0.5	0.4	0.6	0.5
18:3ω6	0.9	1.4	8.5	8.1	11.0	10.2	0.4	0.9
18:3ω3	2.3	2.2	+	+	1.3	1.0	3.6	4.0
18:4ω3	53.1	69.9	49.5	57.0	52.3	62.3	57.9	54.2
18:5ω3	37.1	12.6	34.3	16.6	30.2	11.1	27.7	4.6
20:4ω6	-	-	-	-	-	-	1.3	0.5
20:5ω3	+	0.8	0.8	4.9	1.5	8.3	0.3	1.8
22:6ω3	+	8.3	+	6.5	+	2.2	+	7.3

[a]A + sign indicates that the acid was detected but comprised less than 0.2% of the total fatty acids.

TABLE IV

Major Fatty Acids of Axenically Grown Dinoflagellate

	Gymnodinium microadriaticum			*Amphidinium klebsii*			*Amphidinium carterae*		
	Total lipid	MGG	DGG	Total lipid	MGG	DGG	Total lipid	MGG	DGG
Acid									
14:0	11.5	2.4	2.2	2.2	1.5	0.6	1.9	1.2	0.3
16:0	33.0	13.2	9.8	19.1	4.7	2.1	20.2	6.7	2.3
16:1	21.7	19.8	13.9	1.0	0.7	0.2	0.5	0.5	+[a]
16:2	3.7	6.4	6.1	+	+	+	+	+	+
16:3	2.1	7.1	6.8	-	-	-	-	-	-
18:0	1.3	1.3	0.9	4.1	0.2	0.4	4.3	0.3	1.0
18:1	0.2	+	+	15.9	18.6	4.7	3.3	3.6	1.0
18:2ω6	0.7	1.2	0.9	+	+	+	1.9	3.9	1.3
18:3ω6	3.3	8.3	6.2	8.7	15.4	13.0	2.5	5.2	3.3
18:3ω3	+	-	-	0.9	+	+	+	-	-
18:4ω3	9.2	25.6	39.1	21.4	39.9	38.1	24.7	41.2	44.3
18:5ω3	3.7	13.2	8.8	0.5	1.8	0.4	1.7	6.1	0.8
20:5ω3	0.4	1.3	1.7	11.5	7.8	35.4	22.5	28.8	45.0
22:6ω3	7.8	0.2	1.8	10.7	0.6	0.4	15.2	2.4	0.4

[a]A + sign indicates that the acid was detected but comprised less than 0.2% of the total fatty acids.

content still resembles those of zooxanthellae isolated directly from the same host, even though the axenic culture had been maintained for 3 years. In contrast, the lipids of both *Amphidinium* species contain only low levels of 18:5 which may explain why the acid has not been detected in previous analyses of *A. carterae* lipids (5,11). The high levels of hexadecenoic acid (16:1) in all fractions of the axenic zooxanthellae were not observed in symbiotic zooxanthellae.

DISCUSSION

The results just presented show clearly that zooxanthellae isolated from different hosts may have quite different fatty acid compositions. This observation raises a number of questions. The first concerns the mechanism of synthesis of polyunsaturated fatty acids in the cell, especially when it is in symbiosis with its host. Joseph (6) suggested that 18:5 might be synthesized by removal of a 2-carbon unit

from 20:5, and divided a number of dinoflagellates into 2 groups according to the ratio of 18:5 to 22:6 in their total fatty acids. From the data in Table I, it is difficult to place zooxanthellae into either group as the ratio of 18:5 to 22:6 varies from 0.5 to 1.1. It is true, however, that those zooxanthellae with the highest contents of 18:5 (i.e., those from clams) have the lowest levels of 20:5 and this fact might indicate a precursor-product relationship. But, if this is the case, it is difficult to envisage why the postulated conversion of 20:5 to 18:5 does not go to completion in coral zooxanthellae. The presence of 18:3ω6 in zooxanthellae also is of interest. This isomer is characteristic of animal tissues (12) but its presence in the galactolipids obviates the possibility that it might be a contaminant. The normal isomer of 18:3 occurring in plants is 18:3ω3, which is the major component of the fatty acids of chloroplast galactolipids in higher plants (13).

The level of 18:3ω6 in a total extract of *A. millepora* (i.e., polyp + zooxanthellae) is lower than that in isolated zooxanthellae, indicating that the acid is concentrated in the symbiont.

Apart from the possibility that the differences in fatty acid composition of zooxanthellae from different hosts result from an effect of the host on the metabolism of the symbiont or to translocation of fatty acids from host to symbiont, it is possible that the organisms infecting corals and clams are not identical. It has been widely assumed that the same species *Gymnodinium microadriaticum* infects both clams and corals (1) but recently Schoenberg and Trench have suggested, on the basis of biochemical, ultrastructural and infectivity differences in axenic cultures of zooxanthellae isolated from a variety of marine invertebrates, that different strains of zooxanthellae exist with varying abilities to infect different hosts (2-4). In that case, our results could be explained on the basis of differing strains of dinoflagellates infecting clams and corals. It is apparent also that the fatty acid composition of zooxanthellae isolated from the coral *P. damicornis* (family Pocilloporidae) is significantly different from that of the organisms infecting the 5 species of *Acropora* (family Acroporidae) and the 2 species of the family Faviidae. The high content of 18:4 in *P. damicornis* zooxanthellae is more similar to that of clam zooxanthellae than to coral, but like other coral zooxanthellae, *P. damicornis* contains more 18:4 in MGG than DGG. The lipids of the axenic culture of zooxanthellae from *T. maxima* resemble those of the organism isolated directly from the host, with respect to the low content of 20:5 and the fact that DGG contains more 18:4 than MGG. This finding, together with the lack of 18:5 and low levels of 18:3ω6 in clam tissue, argues against the translocation of fatty acids from host to symbiont. Further evidence in this direction will, however, require the isolation of axenic cultures of zooxanthellae from different hosts, but if different strains of organisms do infect different hosts, then lipid analysis may provide a rapid and reliable means of identifying differences between the great number of zooxanthellae which infect marine invertebrates.

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Bile Acids: LXI. Synthesis and Properties of Conjugates of 5α-Bile Acids

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ABSTRACT

Allo bile acids and their taurine- and glycine-conjugates were synthesized by modification of existing procedures. Their chromatographic and spectral properties were investigated and compared with the 5β-analogs. Some of the naturally occurring 5α- and 5β-conjugates were separable by high performance liquid chromatography via straight-phase (Corasil II column) or reverse-phase systems (μBondapak/C_{18} column), though unsuccessful with thin layer or gas liquid chromatography. Differences between the C-5 epimers in the infrared and proton magnetic resonance spectra could be attributed to the configurations at the A/B ring junction and the configuration of the 3-hydroxyl group. The calcium salts of the allo compounds were generally less soluble in water than the 5β-isomers.

ABBREVIATIONS

T, Tauro; G, glyco; A, allo or 5α-; C, cholate (3α,7α,12α-trihydroxy-5β-cholanate); DC, deoxycholate (3α,12α-dihydroxy-5β-cholanate); CDC, chenodeoxycholate (3α,7α-dihydroxy-5β-cholanate); LC, lithocholate (3α-hydroxy-5β-cholanate); TLC, thin layer chromatography; PLC, preparative layer chromatography; GLC, gas liquid chromatography; HPLC, high pressure liquid chromatography; PMR, proton magnetic resonance; MS, mass spectrometry; IR, infrared; EEDQ, N-ethoxycarbonyl-2-ethoxy-1,3-dihydroquinoline.

INTRODUCTION

The occurrence of conjugated 5α- or allo bile acids apparently is limited to lower forms of vertebrates such as amphibians and reptiles (1,2), in contrast to the 5β-analogs which are much more widely distributed in mammals. Glycoallodeoxycholate, the first conjugated allo bile acid to be isolated and characterized, was obtained from gallstones of rabbits fed a diet containing 5α-cholestanol (3). The calcium salt of this allo compound was less soluble than that of calcium glycodeoxycholate, thus accounting for its presence in gallstones. More recently, several conjugated allo bile acids have been found in mammals including man (4-6). To ascertain whether the poor solubility of glycoallodeoxycholate was characteristic of only this analog of deoxycholate (e.g., choleic acid formation [7]) or was a characteristic generally of the coplanarity of the nucleus of the allo acids, the synthesis of glycine and taurine conjugates of several allo bile acids was undertaken and their chromatographic and spectroscopic properties and solubilities were determined and compared with their 5β-analogs.

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MATERIALS AND METHODS

Commercial samples of 5β-bile acids were purified via the methyl esters by established methods. Methyl allocholate and allochenodeoxycholate and their 3β-isomers were obtained from the respective 5β-isomers by Raney nickel allomerization as described previously (8). In addition, 3α-hydroxy allo bile acids were prepared from their 3β-isomers by a new method. Chromatographic standards of sodium salts of conjugated 5β-bile acids were obtained from Supelco Inc. (Bellefonte, PA). Taurine and glycine ethyl ester hydrochloride were products of Sigma Chem. Co. (St. Louis, MO).

Acetone, benzene and hexane were distilled. Anhydrous methanol was Mallinckrodt analytical grade. Absolute ethanol was USP reagent grade. Tetrahydrofuran and diglyme were dried by refluxing over sodium and distilled under nitrogen. All other solvents and acids were Fisher ACS certified and ACS reagent grades, respectively. d_4-Methanol originated from Merck Chem. Div. (Darmstadt). IR spectra, MS and melting points (mp) were determined as reported (9); TLC on Silica Gel H or $HF_{254+366}$, PLC, GLC of methyl esters of bile acids and their trimethylsilyl derivatives, and HPLC were carried out as described (9-13). PMR spectra were recorded as described (14) in d_4-methanol. Elemental analyses were performed by Galbraith Lab. (Knoxville, TN).

Preparation of 3α-Hydroxy Allo Bile Acids from Their 3β-Epimers

Epimerization of the 3β-tosylate according to Baker et al. (15). Methyl 3β,7α-dihydroxy-5α-cholanate (8) was tosylated, epimerized with

tetra-*n*-butylammonium acetate, and the product hydrolyzed, remethylated and purified by PLC to yield methyl 3α,7α-dihydroxy-5α-cholanate in an overall yield of 56%. Upon alkaline hydrolysis, allochenodeoxycholic acid was obtained with mp 244-246 C (reported [11] mp 245-246 C).

Epimerization of the 3β-hydroxyl group via the methods of Bose et al. (16) and Herz et al. (17). A mixture of methyl 12α-hydroxy-3-oxo-5α- and 5β-cholanates from the Raney nickel allomerization of methyl deoxycholate (8) was reduced in dry diglyme with lithium aluminum tri-*t*-butoxy hydride to provide a product which was separated by PLC into methyl 3β,12α-dihydroxy-5α-cholanate (60%) and methyl 3α,12α-dihydroxy-5β-cholanate (20%). After further purification of the 3β,12α-diol by grade III alumina column chromatography, it was stirred overnight with a mixture of triphenylphosphine, benzoic acid and diethyl azodicarboxylate (16,17) in dry tetrahydrofuran. After hydrolysis and purification by chromatography on grade V alumina and crystallization, allodeoxycholic acid (8) (pure by TLC, and by GLC as the methyl ester on 3% OV-210) was obtained in an overall yield of 49%.

Similar treatment of a mixture of methyl 3-oxo-5α- and 5β-cholanates obtained from allomerization of methyl lithocholate provided colorless crystals of allolithocholic acid (49% overall yield) comparable in mobility in GLC as its methyl ester (3% OV-17) and mp to authentic allolithocholic acid (18). Methyl 3β,7α-dihydroxy-5α-cholanate was epimerized and hydrolyzed similarly to provide a 46% yield of allochenodeoxycholic acid.

General Procedure for the Preparation of the Sodium Salts of Taurine and Glycine Conjugated Bile Acids

A mixture containing 1 mmol of bile acid, EEDQ (1.4 mmol) and taurine (1 mmol) in abs. ethanol (30 ml/mmol of bile acid) was treated with one mmol of 0.25 N sodium hydroxide in abs. ethanol. After refluxing for 2 hr, more EEDQ (1.4 mmol) was added, and the refluxing was continued for another 1.5 hr. The reaction mixture was then worked up as described by Lack et al. (19). Yields of 85-90% were obtained for conjugates such as tauroallocholate, taurochenodeoxycholate and taurodeoxycholate. Anal. NaTAC 1 1/2 H_2O: calcd: C 55.35%, H 8.40%, N 2.48%, S 5.68%, Na 4.07%; found: C 56.02%, H 8.40%, N 2.27%, S 5.07%, Na 4.15%. NaTACDC $3H_2O$: calcd: C 54.24%, H 8.75%, N 2.43%, S 5.57%, Na 3.99%; found: C 54.13%, H 8.49%, N 2.74%, S 6.02%, Na 4.63%. Sodium content was determined by atomic absorption spectroscopy. No calcium was detectable with this instrument. No ninhydrin-positive material was present in the sample; no free bile acid or EEDQ was observed in TLC or HPLC.

Alternately, a mixture of a bile acid (1 mmol), EEDQ (1 mmol) and glycine ethyl ester (1.5 mmol) (19) in anhydrous ethyl acetate (50 ml) was refluxed with stirring for 5.5 hr under anhydrous conditions. After addition of a second mmol of EEDQ, the refluxing was continued overnight. Work-up of the reaction mixture (19) gave an average yield of ca. 90% for glycodeoxycholate and glycoallochenodeoxycholate and 64% for glycoallocholate. Methyl esters of glyco-conjugates were prepared using 2,2-dimethoxypropane (20,21).

RESULTS AND DISCUSSION

Chemistry

Allodeoxycholic and allolithocholic acids were prepared from the corresponding 3-oxo precursors by a new route to bypass the difficulties encountered earlier (2) in the separation of the C-5 epimers of 3-oxo bile acid methyl esters obtained from Raney nickel allomerization of the 5β-cholanates. The components of the mixture were reduced in good yield to the corresponding equatorial 3-hydroxyl products which were then separated by careful PLC. The 3β-hydroxy-5α-cholanate thus obtained could be epimerized either via the tosylate (15) or with diethyl azodicarboxylate and triphenylphosphine (16,17) to the corresponding 3α-isomer as illustrated with methyl 3β,7α-dihydroxy-5α-cholanate. Although the yields were comparable in both these methods for the bile acid studied, the second procedure was preferred because of a shorter reaction time. However, several side products were observed in this reaction; the major one apparently was an unsaturated compound as judged by its mobility in GLC.

Conjugated allo bile acids were prepared by the Lack et al. method (19). By maintenance of anhydrous conditions and addition of a second batch of EEDQ after the initial reaction period, the yields of the crystallized products were increased to ca. 85-90%. A single crystallization was sufficient to remove unreacted material; the monohydroxy derivatives were crystalline, but the others were amorphous. Since completion of these studies, Tserng et al. (22) have reported an alternate procedure.

Table I includes melting points, and the mobilities of the synthetic conjugated allo bile acids on TLC and HPLC. Despite occasional

TABLE I

Properties of Some Conjugated 5α-Bile Acids

	mp (°C)	R_f[c]	R_f[d]	R_f[e]	RR_V[f]	RR_V[g]
TAC[a]	302-305	0.50	0.28	0.43	0.62	0.47 (0.47)
TADC[a,b]	265-280	0.63	0.39	0.58	0.87	0.86 (0.90)
TACDC[a]	210-212	0.60	0.39	0.57	0.85	0.75 (0.75)
TALC[a]	250-260	0.72	0.51	0.69	1.41	1.65 (1.60)
GAC[a]	283-287	0.36	0.06	0.28	1.14	0.46 (0.46)
GADC[a]	261-264	0.51	0.14	0.42	2.23	0.82 (0.88)
GACDC[a]	230-232	0.51	0.15	0.44	1.95	0.74 (0.73)
GALC[a]	256-269	0.62	0.20	0.53	–	1.55 (1.50)

[a]T denotes tauro-; G, glyco-; A, allo; C, cholate; DC, deoxycholate; CDC, chenodeoxycholate; LC, lithocholate. Thus, TAC refers to tauroallocholate.

[b]Sample turned yellow upon heating.

[c]Solvent system: $CH_3OH/CHCl_3/H_2O$ (15:20:2, v/v).

[d]Solvent system: 2-propanol/$CHCl_3$/Conc. NH_4OH (30:10:1, v/v).

[e]Solvent system: $CH_3OH/CHCl_3$/7 N NH_4OH/H_2O (15:30:1:2, v/v).

[f]Relative Retention Volume (RR_V) related to taurodeoxycholate = 1; eluting solvent: 2-propanol/8.8 mM potassium phosphate buffer, pH 2.5 (160:340); elution rate = 1 ml/min through a "fatty acid analysis" column (30 cm × 4 mm id; Waters Associates) (13).

[g]Solvent: 2-propanol/10 mM potassium phosphate buffer, pH 7.0 (160:340); elution rate = 1 ml/min through a μBondapak/C_{18} column (30 cm × 4 mm id Waters Associates) (26). Values in parentheses are those of the corresponding 5β-bile acid conjugates. The chromatographic standard is deoxycholic acid.

wide ranges in mp, the purity of these preparations was shown by their homogeneity in the chromatographic systems. The glycine conjugates were further derivatized as methyl esters and their chromatographic purity was established in several systems (see following).

Chromatography

Separation by TLC of these conjugates from their 5β-analogs is difficult and laborious, but small differences (ca. 0.02 in R_f values) in their mobilities are observed on plates of Silica Gel H in the solvent system methanol/chloroform/7 N ammonium hydroxide/water (15:30:1:2, v/v), a modification of system I of Subbiah and Kuksis (23). Multiple developments were helpful for the separation of GDC (R_f 0.35) and GADC (R_f 0.39) (one and one-half developments) and GC (R_f 0.25) from GAC (R_f 0.23) (4 full developments) on EM "high performance silica gel 60" TLC plates in the solvent system *i*-amyl alcohol/acetic acid/water (18:5:2, v/v). Separation of the positional isomeric dihydroxyl derivatives by TLC traditionally has been a problem. Eastman chromatogram sheets appear useful in limited separations of pairs of dihydroxy conjugates such as GDC (R_f 0.14) and GCDC (R_f 0.16), TACDC (R_f 0.38) and TADC (R_f 0.40), and GACDC (R_f 0.16) and GADC (R_f 0.14) with the solvent system 2-propanol/chloroform/7N ammonium hydroxide (30:10:1, v/v). The proximity of the taurine and glycine conjugates of the 5β- and 5α-bile acids with this solvent mixture has been reported (24).

Some improvement in separation of pairs of C-5 epimers of these conjugated acids can be found with HPLC. With a Waters Associates Model 201 HPLC system (12) and a "fatty acid analysis" or a μBondapak/C_{18} column, relative retention volume (RR_V) of conjugated allo bile acids were essentially similar to those for the 5β-derivatives with a solvent system of 2-propanol/8.8 mM potassium phosphate buffer (pH 2.5) or 2-propanol/10 mM potassium phosphate buffer (pH 7.0), respectively, in a ratio of 160:340 (Table I). However, the conjugates of 5β- and 5α-deoxycholate are partially separable (Fig. 1) on one pass through the column.

The ability of straight-phase HPLC systems to separate epimers by recycling through the column is shown in Figures 2 and 3 in which the epimeric glycocholates were resolved in 3 cycles and the taurocholates in 5 or 6 cycles on 5 sequential columns of Corasil II with systems of acetonitrile/acetic acid/water (400:20:5) and ethyl acetate/2-propanol/water/7N ammonium hydroxide (900:300:100:0.5), respectively. Since TC and TAC occur naturally in significant quantities (1,2,24,25), this procedure offers a useful method to detect and separate TAC. Similar separations of GDC and GADC, and GLC and GALC have been reported (26). Partial separation of GCDC and GADC have

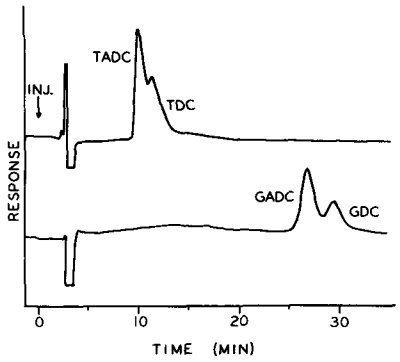

FIG. 1. HPLC separation of TDC and TADC, and GDC and GADC on a "fatty acid analysis" column (Waters Associates) in solvent system 2-propanol/8.8 mM potassium phosphate, pH 2.5 (160:340) at a flow rate of 1 ml/min; loop injection; differential refractometer as detector. Separation of TDC and TADC could conceivably be improved by recycling.

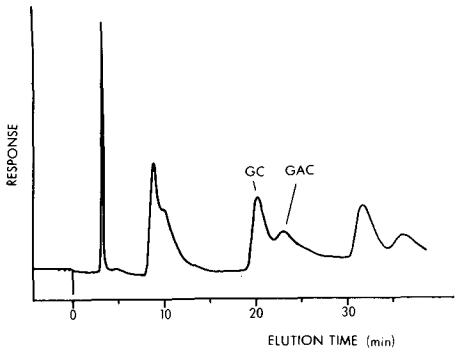

FIG. 2. HPLC separation of GC by the recycling mode (retention volume during the first cycle, R_V, 17.6 ml) and GAC (R_V, 20 ml) on 5 2-ft columns of Corasil II connected in series, in solvent mixture acetonitrile/acetic acid/water 400:20:5; flow rate 2 ml/min; stop-flow septum injection; differential refractometer as detector.

been obtained after 5 cycles with a mixture of acetonitrile/acetic acid (400:10). Partial resolution of pairs of taurine conjugates (e.g., TLC and TALC, TCDC and TACDC) also are observed after several cycles on Corasil II with solvent mixtures of ethyl acetate/2-propanol/water (300:100:15) and ethyl acetate/2-propanol/7 N ammonium hydroxide (300:100:25:0.5), respectively. Experience with a μPorasil column (30 cm x 3.9 mm id) suggests that similar separations of the glycine conjugates can be effected with the solvent system(s) just described instead of the use of 5 sequential columns of Corasil II.

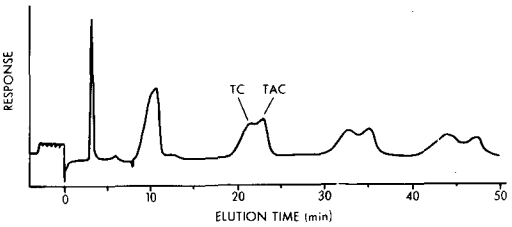

FIG. 3. HPLC separation of TC (R_V 19.3 ml) and TAC (R_V 21.4 ml) by the recycling mode on 5 Corasil II columns in a mixture of ethyl acetate/2-propanol/water/7 N ammonium hydroxide (900:300:100:0.5); other conditions as in Fig. 2.

Solubility of Salts

Hofmann and Mosbach (3) showed that the aqueous solubility of the calcium salt of glycodeoxycholate was far greater than that of calcium glycoallodeoxycholate. The Hofmann and Mosbach experiment (3) was extended as follows (Fig. 4): to 0.5 ml of 0.04 M solution of the conjugate, aliquots of a solution of 0.04 M calcium chloride were added. Precipitates were observed in solutions of GDC and GADC with 0.725 ml and 0.005 ml, respectively, whereas solutions of GCDC and GACDC required 0.40 ml and 0.05 ml of the calcium chloride solution, respectively. When TACDC was used, 0.7 ml caused precipitation, whereas with TCDC, GC and GAC, TC and TAC no precipitate was noted after 11.5 ml of calcium chloride solution were added. The sodium salts of the monohydroxy GLC and GALC are so insoluble that no data were obtained with these derivatives. The poor solubility of the monohydroxy bile acids is illustrated by the

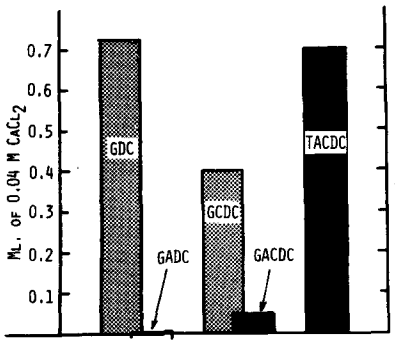

FIG. 4. Comparative solubilities of calcium salts of some conjugates of allochenodeoxycholate and allodeoxycholate and their 5β-analogs. GDC = glycodeoxycholate; GADC = Glycoallodeoxycholate; GCDC = glycochenodeoxycholate; GACDC = glycoallochenodeoxycholate; TACDC = tauroallochenodeoxycholate. Height of the bars represents the ml of 0.04 M $CaCl_2$ required to form a precipitate with 0.5 ml of a 0.04 M solution of the conjugate. TCDC failed to precipitate after addition of 11.5 ml of the $CaCl_2$ solution.

TABLE II

Infrared Spectral Absorption Maxima (cm^{-1}) of Sodium Salts of Conjugated Trihydroxy Bile Acids

TC[a]	3215-3300	–	1198	1170	1049	976	–	943	–	909	–
TAC	3279	–	1198	1170	1042	–	1010	–	955	–	888
GC	3279	1597	–	1157-1164	1031-1042	976	–	942	–	909	–
GAC	3259	1590	–	1147-1160	1027-1038	–	1008	–	955	–	888

[a]See Table I for explanation of symbols.

following: 10 mg of sodium TLC require 11 ml of water for solubilization whereas sodium TALC required 30 ml; the aqueous mixture containing solid bile acid was shaken occasionally over a one-week period with incremental addition of water until no solid remained. Since the solution of calcium chloride was acidic, these comparative data should not be considered as representative of physiological conditions, for neither temperature, ionic strengths, pH nor concentrations were comparable to physiological circumstances. However, these observations confirm and extend those of Hofmann and Mosbach to show that the conjugates and their calcium salts of several of the more planar allo bile acids are generally much less soluble than the corresponding 5β-derivatives.

Spectral Analyses

Supportive evidence for identification of substances eluted by HPLC in a particular fraction can be provided by MS, and occasionally by IR and PMR. Mass spectral differentiation of the taurine conjugates, and the glycine derivatives and their methyl ester have been reported (27). With exception of the pair TC and TAC, all other pairs can be distinguished by examination of at least one of the major fragmentations. The IR spectra of the sodium salts of conjugated allo bile acids are distinguishable from the analogous 5β-derivatives by absorption bands exhibited by nuclear hydroxyl groups in the vicinity of 1031, 1010, 960 and 890 cm^{-1} (1,2,18). The taurine conjugates exhibited absorption at 1200 cm^{-1} for the sulfonate anion and the glycine conjugates showed absorption in the vicinity of 1595 cm^{-1} for the carboxylate anion. Table II compares characteristic absorption bands for the taurine and glycine conjugates of cholate and allocholate.

PMR spectra were predictably useful in differentiating the C-5 epimers by virtue of the chemical shifts of the protons at C-3 and C-19 as exemplified by the pairs TC-TAC and GC-GAC in Table III, analogous to the observations of Small et al. (28) and Shalon and Elliott (29) for the free bile acids. Glycine conjugates were characterized by chemical shifts of the protons of the amino acid moiety (δ=3.74), whereas the taurine derivatives exhibited a shift at 2.96 and 3.60.

Methyl esters of the 8 glycine conjugates were converted to their trimethylsilyl ethers (TMS), and chromatographed on 3% OV-17 at 260 C. Retention times relative to the bis-TMS ether of methyl deoxycholate for the 8 derivatives were 0.58 or 0.59; thus, failure to resolve these derivatives is in accord with an earlier observation of Hanaineh and Brooks (30). Treatment of the taurine conjugates with diazomethane, boron trifluoride/methanol, methyl iodide/dimethyl sulfoxide, 2,2-dimethoxypropane/HCl, diazomethane/pyridine or methanol/HCl did not provide sufficient amounts of a less polar product with characteristics of a methyl ester to warrant study by GLC.

In view of these chemical and physical differences between the C-5 epimeric conjugated bile acids, it will be of interest to study their biochemical and physiological properties. The frequent and sometimes predominant occurrence of the 5α-compounds in lower forms of animals in contrast with the predominance of the 5β-analogs in the more highly evolved vertebrates confers much significance on such studies in affording information on the evolutionary role of these epimers.

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TABLE III

Proton Magnetic Resonance Absorptions (δ,ppm) of Sodium Salts of Conjugated Trihydroxy Bile Acids in d_4-Methanol with a Varian *HA 100* Spectrometer

	18-H(s)	19-H(s)	21-H (d;J=5 Hz)	N-CH_2-C- (Tauro) (t;J=7 Hz)	CD_3OD	C-CH_2-SO_3Na (Tauro) (t;J=7 Hz)
TC[a]	0.69	0.90	1.02	2.96	3.34	3.60
TAC	0.68	0.78	1.01	2.97	3.32	3.60
GC	0.70	0.90	1.04	–	3.30	–
GAC	0.69	0.79	1.03	–	3.31	–

	N-CH_2-CO_2Na (Glyco) (s)	3-H (m)	7-H (m)	12-H (m)
TC	–	masked	3.81	3.96
TAC	–	3.98	3.80	3.94
GC	3.74	3.42	3.81	3.97
GAC	3.74	3.98	3.80	3.94

[a]See Table I for explanation of symbols.

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Lecithin-cholesterol Acyltransferase: Inhibition by Local Anesthetics in Plasma from Man and Experimental Animals in vitro

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ABSTRACT

Lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43) was assayed in vitro in plasma from normal man, rat, rabbit and dog by following the formation of ^{14}C-cholesteryl esters subsequent to labeling the plasma with ^{14}C-cholesterol in vitro. In all species examined, various local anesthetics were found to inhibit LCAT when studied over the concentration range of 1 to 5 mM. The order of inhibition was dibucaine>benzocaine>tetracaine>lidocaine>procaine. Since LCAT activity represents the combined effect of a deacylation step and an esterification step, inhibition of LCAT by local anesthetics could theoretically involve either or both steps.

INTRODUCTION

Lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43) occurs in the plasma of man and other animals (1-4) and catalyzes the esterification of cholesterol with fatty acid derived from the 2-position of lecithin (5,6); high density lipoprotein (HDL) lecithin is the preferred substrate (2). The overall action of the enzyme is achieved via the integration of a lipase and a transferase step which can be distinguished as 2 separate events (7-9). Although the full physiological significance of LCAT is not understood in species such as man, LCAT activity contributes significantly to the circulating level of plasma steryl esters (10) and influences the metabolism of plasma lipoproteins as well (11-15).

MATERIALS AND METHODS

Human blood from normal male volunteers (21-28 years old) was drawn into tubes containing sodium citrate, and blood from normal male laboratory animals was drawn into heparinized tubes. The animals used were 3-5-yr-old mongrel dogs, New Zealand rabbits (2.5-3 kg) and Sprague-Dawley rats (Upj:TUC(SD)spf) (200-250 g). The plasma was separated immediately by centrifugation at 4 C and assayed for LCAT activity using [4-^{14}C] cholesterol (New England Nuclear Corp.; sp act 54 mCi/mM) and measuring the formation of ^{14}C-cholesteryl esters.

LCAT Assay

LCAT activity was assayed in human plasma by the Stokke and Norum method (16) as modified by Lacko et al. (17). In this method, plasma in which LCAT has been temporarily inhibited by 5,5-dithiobis-2-nitrobenzoic acid (DTNB, final concentration 0.7 mM), is preincubated for 4 hr with albumin-stabilized isotopic cholesterol. During the preincubation period, isotopic cholesterol transfers from the albumin to the plasma lipoproteins and yields ca. 90% equilibration with endogenous lipoprotein cholesterol (16,17). The assay is initiated by adding β-mercaptoethanol (final concentration, 11.8 mM) to eleviate the inhibition of LCAT. In addition, LCAT was assayed without preincubation in plasma samples to which ^{14}C-cholesterol was added directly (0.7 μCi/ml plasma) as a solution in 50 μl acetone (18,19). This method has been used to measure net esterification of cholesterol (19) whereas a method involving preincubation of plasma with protein-bound isotopic cholesterol as just outlined (17) permits the additional measurement of initial rates of esterification. The 2 methods were used to assay LCAT in human plasma in this study; since both were found to be equivalent in reflecting the relative potency of LCAT inhibitors, assay of LCAT by the direct addition of isotopic cholesterol in acetone was the method adopted for most of these studies. All assays were conducted at 37 C in a shaking water bath. Plasma samples were removed at various times and extracted with 20 vol of chloroform/methanol (2:1, v/v) (17). The lipid extracts were fractionated by thin layer chromatography (TLC) on glass plates coated with Silica Gel G (20) and the esterified cholesterol fraction was scraped into vials for radioactive assay by liquid scintillation counting (21). In some experiments (human plasma), the unesterified cholesterol was eluted from the silica gel with chloroform

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(21) and measured by gas liquid chromatography (GLC) (22).

All chemicals used were obtained commercially (DTNB, Aldrich Chemical Co., Rochester, NY; β-mercaptoethanol, Eastman Kodak Co., Rochester, NY; benzocaine, dibucaine•HCl, procaine•HCl and tetracaine•HCl, Sigma Chemical Co., St. Louis, MO; lidocaine•HCl, Sterling Organics, New York, NY).

RESULTS

Figure 1 shows the time-course for cholesterol esterification by LCAT in normal human plasma employing the Lacko et al. assay method (17). Esterification was essentially linear over the entire 60-min assay interval. Equilibration of the ^{14}C-cholesterol was essentially complete prior to initiation of the assay (0 time) since unesterified cholesterol specific activity (sp act) measured in triplicate at 0 time and 60 min was 1098 ± 7 and 1114 ± 14 dpm/mμMol, respectively. Data from Figure 1 also clearly show that LCAT is inhibited by addition of lidocaine and tetracaine to the plasma (final concentration, 3 mM). Calculation of initial rates of esterification from the data indicate 0.76 and 0.32 mμMol/ml/min in the presence of lidocaine and tetracaine, respectively, and 1.0 mμMol/ml/min in incubations without additions (control). These data are based on the assumption that there is complete equilibration of labeled exogenous cholesterol with the endogenous cholesterol pool used by LCAT (16,17). Figure 2 presents results of a similar experiment to that illustrated in Figure 1 except that the ^{14}C-cholesterol was added directly to the plasma (in 50 μl acetone) to initiate the assay. The results of Figure 2 confirmed the inhibitory effect of lidocaine and tetracaine and accurately reflected their relative potency. Because of this close agreement between the 2 methods for studying inhibition of LCAT, the direct addition of ^{14}C-cholesterol was used for all subsequent studies reported here.

The data of Figure 3 show that inhibition of LCAT by lidocaine is not species-specific and is concentration-dependent. The inhibition of plasma LCAT in man, dog and rabbit was similar over the concentration range 1-5 mM with ca. 50% inhibition occurring at 5 mM. Rat plasma LCAT apparently was somewhat more sensitive, with 50% inhibition occurring at 3 mM and reaching ca. 70% at 5 mM.

Figure 4 shows the effects of a number of other local anesthetics on plasma LCAT in 3 species. All agents were present at a level of 5 mM. In man, dog and rabbit, the greatest inhibition of LCAT (80-96%) occurred in the presence of dibucaine. Procaine, which was not studied in human plasma, was the weakest inhibitor in dog and rabbit, giving ca. 15% inhibition. Tetracaine was 3-8 times more effective than lidocaine as an inhibitor of

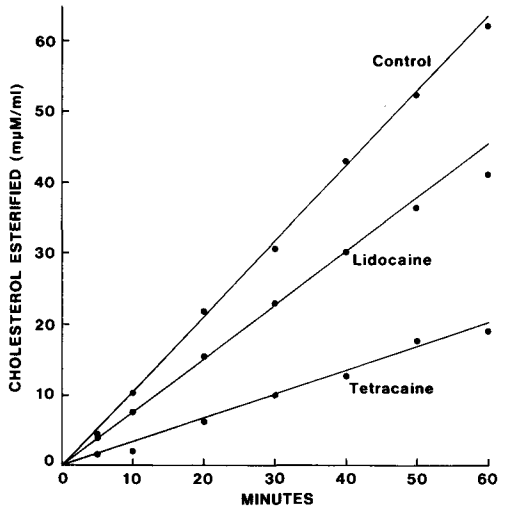

FIG. 1. Time-course of cholesterol esterification in human plasma. Plasma from a normal 21-yr-old male (unesterified cholesterol, 37 mg%) was preincubated with DTNB and albumin-stabilized ^{14}C-cholesterol in order to label the lipoprotein cholesterol. The LCAT assay was initiated by adding β-mercaptoethanol to reverse the DTNB-inhibition of LCAT (see methods). Lidocaine•HCl and tetracaine•HCl were added as saline solutions (168 μl) to 4.5 ml of plasma preparation to yield final concentrations of 3 mM. ^{14}C-Cholesteryl ester formation was measured in 0.5-ml samples taken after various incubation intervals.

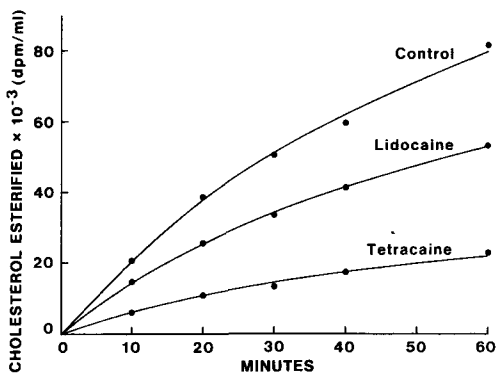

FIG. 2. Time-course of cholesterol esterification in human plasma from a normal 28-yr-old male (unesterified cholesterol, 33 mg%). ^{14}C-Cholesterol (6.7 X 10^6 dpm) in 50 μl acetone was added directly to 4.5 ml of plasma and ^{14}C-cholesteryl ester formation was measured in 0.5-ml samples taken after various incubation intervals.

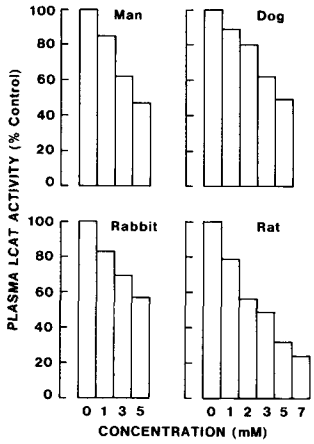

FIG. 3 The effect of lidocaine on plasma LCAT activity in normal man, dog, rabbit and rat in vitro. Plasma LCAT activity was assayed by measuring the formation of ^{14}C-cholesteryl esters in 2 hr at 37 C subsequent to the direct addition to the plasma of ^{14}C-cholesterol dissolved in acetone (see Methods). ^{14}C-Cholesteryl ester formation in plasma samples containing lidocaine is expressed relative to control samples assayed in the absence of lidocaine.

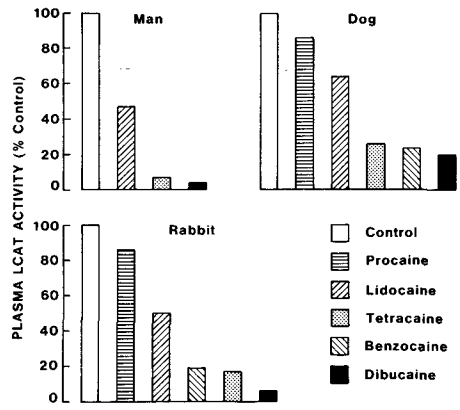

FIG. 4. A comparison of the effects of various local anesthetics on plasma LCAT activity in normal man, dog and rabbit in vitro (see footnote to Fig. 3). All agents were present at a final concentration of 5 mM.

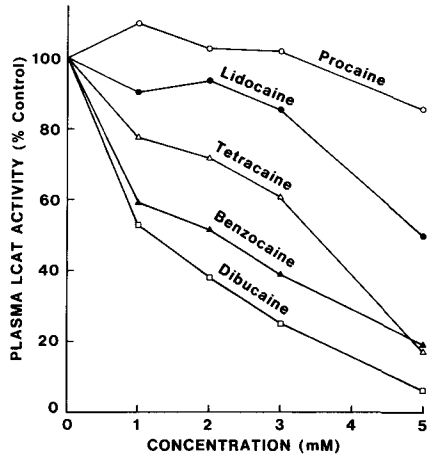

FIG. 5. A comparison of the concentration-dependent effect of various local anesthetics on plasma LCAT activity in the rabbit in vitro (see footnote to Fig. 3).

LCAT in the 3 species. Benzocaine was similar to tetracaine in potency when compared in dog and rabbit plasma.

In Figure 5, 5 local anesthetics were studied in rabbit plasma over the concentration range 1-5 mM. LCAT inhibition was in the order dibucaine>benzocaine>tetracaine>lidocaine>procaine. On a molar basis up to a level of 3 mM, benzocaine was ca. 20% more potent than tetracaine; these 2, however, exhibited almost identical potency at 5 mM. A similar observation was made in dog plasma (Fig. 4).

DISCUSSION

This study presents evidence that various local anesthetics are inhibitors of plasma LCAT in man and experimental animals. The inhibition is not species-specific but there may be some species differences in sensitivity of LCAT to the inhibitors. Although the mechanism of the inhibition was not studied, it is possible that the anesthetics inhibit LCAT by interfering with one or both of the coupled activities of LCAT which consists of deacylation of lecithin by a phospholipase A_2-like activity and an esterification of the acyl group with cholesterol (7-9). Inhibition of either step is within the realm of known effects of local anesthetics such as the inhibition of phospholipases A and C (23-26), the inhibition of hormone-stimulated lipolysis (27), and the inhibition of cholesterol esterase (28). If the LCAT reaction proceeds through an acyl-enzyme intermediate (9), a possible mechanism of the local anesthetics could be in destabilizing, or promoting hydrolysis of, the intermediate. A further point to be made is that inhibition of LCAT by the various local anesthetics does not necessarily indicate a similar mechanism of action for all. For example, the inhibition of mitochondrial phospholipase A_2 by dibucaine may reflect an interaction with the enzyme (24) whereas inhibition of pancreatic phospholipase A_2 by dibucaine may be via an interaction with substrate (26). A binding of local anesthetic to phospholipids (26,29,30) or other constituents

of substrate HDL could conceivably affect LCAT activity by modifying the fluidity of HDL lipids. Furthermore, the effect of a local anesthetic on an enzyme system may be variable (stimulatory or inhibitory) depending on such factors as anesthetic concentration (see procaine, Fig. 5) and the physical state of the substrate (23,24,26). Displacement of Ca^{++} has also been suggested as the mechanism of enzyme inhibition by some local anesthetics (23,24). It is unlikely that Ca^{++} displacement is involved in LCAT inhibition since both the deacylation step and transesterification step proceed independently of a Ca^{++} requirement (8,9). Studies employing artificial substrates and isolated LCAT will be necessary to investigate the mechanism of LCAT inhibition.

The relative differences in potency of the different anesthetics on LCAT as observed here is unlikely to reflect differing stabilities of the agents in plasma; we have recently observed a similar rank order of inhibitory potency as shown in Figure 5 in studies of rat hepatic microsomal ACAT (acyl CoA:cholesterol acyltransferase, EC 2.3.1.26) (31).

The interesting observation that plasma LCAT activity is decreased in patients following myocardial infarction (32) should be mentioned here since lidocaine-infusion in man following myocardial infarction is a common medical practice (32,33). Therapeutic concentrations of lidocaine are usually ca. 5 μg/ml plasma (0.02 mM) (33), and, based on the data given here, would not be expected to appreciably affect LCAT activity in man in vivo. The possibility, however, cannot be dismissed without direct evaluation.

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Effect of Chronic Ingestion of DDT on Physiological and Biochemical Aspects of Essential Fatty Acid Deficiency[1]

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ABSTRACT

Male weanling rats were fed semipurified diets with and without essential fatty acid (EFA) and DDT (150 ppm) for 14 weeks to determine the effects of the pesticide on physiological and biochemical aspects of EFA deficiency (EFAD). DDT did not affect EFAD-induced reduction in growth rate or final body weight, nor did the pesticide affect EFAD-induced changes in feed efficiency or skin dermatitis. The pesticide did increase liver/body mass ratios, but did not interact with EFAD, which also increased this ratio. The pesticide produced complex changes in total fatty acid composition of liver and tail skin: liver levels of 18:0, 18:2 and 20:3ω9 were increased, whereas levels of 12:0, 14:0 and 16:0 were decreased. In both tissues, DDT interacted with EFA to increase 18:2 levels. DDT did not change the total fatty acid 20:3ω9/20:4ω6 ratio in either tissue. In this study, although DDT did not exacerbate the physiological aspects of EFAD, DDT-induced changes in fatty acid composition of liver and tail skin indicated that 150 ppm DDT in the diets did alter lipid metabolism of the rats in an unexplained manner.

INTRODUCTION

Essential fatty acid deficiency (EFAD) results in well-characterized deficiency symptoms that have been studied in a variety of species (1). In the rat, the symptoms include scaley dermatitis of the feet and tail skin, necrosis of the tail, decreased growth rates, reduced adult body weights and fatty infiltration of the liver. Lack of dietary linoleic acid (18:2ω6, hereafter referred to as 18:2) resulting in EFAD, produces complex changes in tissue fatty acid composition, including decreased tissue levels of 18:2 and its essential metabolite, arachidonic acid (20:4ω6). The lack of 18:2 is associated with increased conversion of linolenic acid (18:1ω9) to eicosatrienoic acid (20:3ω9). This conversion is inhibited by 18:2 in tissue adequate in EFA (2).

Tinsley and Lowry (3) have reported that ingestion of 150 ppm DDT produced physiological and biochemical changes in EFA-deficient male and female rats over a 12-week period. They observed DDT ingestion to be associated with decreased body weight, increased liver mass (per g body weight), and changed hepatic fatty acid composition, most notably an increased percentage of 20:3ω9 resulting in an increased 20:3ω9/20:4ω6 ratio. The authors concluded that DDT accentuated EFAD in male rats.

This study was undertaken to further examine the interaction of DDT and EFA metabolism in 2 tissues affected by EFAD, liver and tail skin.

MATERIALS AND METHODS

Eight sets of litter mate (4/litter) male weanling Sprague-Dawley rats (Holtzman, Inc., Madison, WI) initially weighing 53-67 g (60 ± 4 g, $\bar{X}$ ± SD) were randomly assigned to 4 test diets, with food and water available ad libitum. During the 14-week feeding period, feed consumption was measured daily, and body weights were recorded weekly.

The 4 semipurified diets used varied in EFA and DDT content as indicated in Table I, and were modeled after diets employed by Tinsley and Lowry (3). The EFA-deficient diets contained 25% by weight hydrogenated coconut oil (HCO). EFA-supplemented diets contained 1.7% corn oil and 23.3% HCO, providing 1% of calories as 18:2. DDT was added to the oil fraction at 150 mg/kg of feed. The composition of the basal portion of the diets is listed in Table II. Diets were mixed biweekly and stored at -20 C to impede the development of rancidity.

The liver and tail skin from each rat were analyzed for total fatty acid composition. Tail skin was selected for study because this tissue prominently displays EFAD dermatitis, and

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TABLE I

Composition of Oil Fraction of Diets

Nutrient	Percentage of diet			
	Diet			
	1 -DDT-EFA	2 +DDT-EFA	3 -DDT+EFA	4 +DDT+EFA
Coconut oil (hydrogenated)[a]	25.0	24.985	23.3	23.285
Corn oil[a]	–	–	1.7[b]	1.7
p,p'-DDT[c]	–	0.015	–	0.015
Totals	25.0	25.000	25.0	25.000

[a]Teklad Test Diets, Madison, WI.

[b]This provides 1.02% linoleic acid, as the corn oil contained 60% 18:2ω6.

[c]1,1,1-Trichloro-2,2 bis (*p*-chlorophenyl) ethane, 99% pure, Analabs, Inc. North Haven, CT.

therefore would presumably demonstrate extrahepatic changes caused by EFAD. Dermatitis of the tail and feet was evaluated using the scoring system described by Holman (1).

Rats were sacrificed by decapitation, the tail skin removed, and livers excised. Tissues were stored at -70 C for subsequent analysis. Liver samples were homogenized in a Waring blender, and total lipids extracted as described by Bligh and Dyer (4). The fibrous nature of tail skin necessitated the following treatments prior to extracting: freeze-drying the tissue, fine mincing with surgical scissors, pulverizing with a mortar and pestle and homogenization in a Waring blender. The total lipids of tail skins were extracted by the Bligh and Dyer method (4). The fatty acids in liver and tail skin were obtained from total crude lipids by saponification with methanolic NaOH (5), and converted to methyl esters by methanolic HCl reflux (6). The esters were analyzed using an F&M Model 810 gas chromatograph (Hewlett-Packard Co., Paramus, NJ). Samples were separated with a 6' × 1/8" stainless steel column packed with 10% SP-2330 on 100/120 mesh Chromosorb W-AW (Supelco, Inc., Bellefonte, PA). After injection of the sample, the instrument was maintained at 185 C for 5 min followed by temperature programmed runs from 185-235 C at 4 C/min. Methyl esters were identified by carbon number plots and by comparison to standards (Nu-Chek-Prep., Elysian, MN). Methyl 20:3ω9 was isolated from the liver of a severely EFAD rat and purified by preparative $AgNO_3$ thin layer chromatography (TLC) and used as a standard (7). Quantitation was done with a Disc-Integrator Model 203 (Disc Instruments, Inc., Santa Ana, CA). The coefficient of variation ($SD/\bar{X} \times 100$) for peaks comprising more than 4% of the sample was < 4%. Data were analyzed as 2 × 2 factorial analyses of variance (8).

RESULTS

Growth Rate, Final Body Weight and Feed Efficiency

Figure 1 indicated that rats fed diets 3 and 4 (+ EFA) gained weight at similar rates and were heavier than rats fed diets 1 and 2 (-EFA), which gained weight at similar rates. DDT had no effect on growth when added to either the EFA-sufficient or EFA-deficient diets. Final body weights were increased an average of 46% (Calculated as: percentage change = [factorial effect mean × 100]/[mean of treatments without the factor] = [143.8 ÷ 100]/[0.5 (311 + 318)] = 46%) by EFA supplementation of the diet, but were unaffected by DDT (Table III). Feed efficiency was defined as g of feed consumed/g final body weight: thus a decrease in the ratio indicates an increased efficiency of conversion of feed to body mass. EFA supplementation increased feed efficiency by an average of 26% (Table III). The effect of DDT on feed efficiency was not significant.

TABLE II

Composition of Basal Diet

Nutrient	Percentage of diet
Glucose monohydrate[a]	47.6
Casein[b]	22.0
H.M.W. salts[b]	4.0
A.O.A.C. vitamin mix[b]	1.0
Supplemental salts	0.4
Totals	75.0

[a]Dextrose monohydrate (technical), Tekland Test Diets, Madison, WI; and Clintose Dextrose, Clinton Corn Processing, Inc., Clinton, IA.

[b]High Protein Casein, Teklad Test Diets, Madison, WI.

[c]57.2 mg/kg $ZnSO_4 \cdot 7H_2O$, 155.4 mg/kg $MnSO_4 \cdot H_2O$ 3.780 g/kg KH_2PO_4.

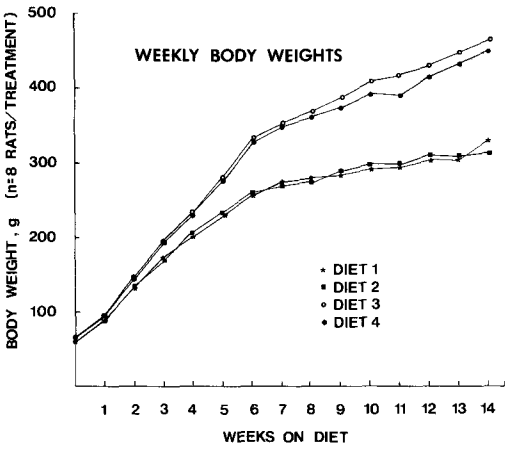

FIG. 1. Gain in body weights of rats on diets. 1: no DDT or essential fatty acid (EFA); 2: DDT, 0.015%, no EFA; 3: no DDT, 1.7% EFA; 4: DDT, 0.015%; EFA, 1.7%.

Liver/Body Weight Ratios

EFA in the diets decreased the liver/body weight ratio by an average of 24%, whereas DDT increased the ratio by an average of 26% (Table III). Analysis of variance indicated no interaction between the dietary factors ($P = .557$).

Dermatitis Scores

The data indicate that EFA supplementation eliminated dermatitis. DDT did not induce or aggravate the condition (Table III). Factorial effect means are listed in Table III.

Liver and Tail Skin Total Fatty Acid Composition

Fatty acid composition of liver and tail skin are presented in Tables IV and V. In liver, DDT increased the percentages of 18:2 and decreased the percentages of 12:0, 14:0 and 16:0. DDT increased hepatic percentages of 18:0 and 20:3ω9 in EFA-deficient animals. Supplementation with EFA increased the percentages of 12:0, 14:0, 16:0, 18:2 and 20:4ω6. DDT and EFA interacted to increase hepatic 18:2, i.e., the weight percentage rise in 18:2 caused by DDT was greater in the presence of EFA (9.1-12.8%) than in its absence (1.4-2.2%). Hepatic 20:3ω9/20:4ω6 ratios were not affected by DDT, whereas EFA supplementation was associated with a fall in the ratio values from 2.2 and 2.7, observed in EFAD rats, to 0.1 DDT increased 18:2 percentages in tail skin (Table V). EFA increased the percentages of 12:0, 14:0, and 18:2 in tail skin, and decreased tail skin 16:0, 16:1, 18:1, 20:1, and 20:3ω9. As in liver, the 2 factors interacted to increase tail skin 18:2 percentages: in the absence of EFA, DDT increased 18:2 from 0.9 to 1.1%, whereas in the presence of EFA, DDT increased 18:2 from 7.1 to 8.4%. As in the liver, DDT did not affect the ratio of 20:3ω9/20:4ω6 in tail skin, whereas EFA supplementation reduced the ratio from EFA-deficient to EFA-adequate levels.

DISCUSSION

This study was designed to investigate the effects of chronic DDT ingestion on several physiological and biochemical aspects of EFA deficiency. The data indicate that while DDT increased liver/body weight ratios and produced changes in the fatty acid composition of liver and tail skin, the pesticide did not have a significant effect on the other parameters studied, including body weight, feed efficiency and the extent of EFAD dermatitis.

Diminution of growth and final body weight have long been recognized as symptoms of EFA deficiency (1). This effect was obvious by the third week in this study, and final body weights of the -EFA rats (14 weeks) were ca. 150 g less than for the +EFA rats. Tinsley and Lowry (3) reported that 150 ppm DDT depressed in EFA-deficient, but not EFA supplemental female rats. No such interaction was observed in male rats. Their observation was confirmed in this study, where inclusion of DDT in the diet did not significantly alter final body weight. EFA-supplemented rats fed DDT did attain slightly lower final weights than did EFA-supplemented rats without the pesticide (452 vs 465 g) but this effect was not statistically significant. Darsie et al. (9) observed reduced 30-day body weights in rats fed 5 or 500 ppm *o,p'*-DDT, and in rats fed 5 ppm *p,p'*-DDT. In this study, a DDT-induced depression of 4-week body weight was not observed (Fig. 1). Berdanier and deDennis (10) have reported no effect of 1 ppm DDT on 200-day weight gains in exercised male rats.

Inclusion of EFA in the diet markedly improved feed efficiency (Table III). Lack of dietary EFA may cause uncoupling of ATP biosynthesis in the mitochondria of deficient rats. Production of ATP in the mitochondria is a membrane-bound phenomenon (11). Because mitochondrial membranes contain considerable amounts of polyunsaturated fatty acids (12), EFA deficiency may cause a derangement of mitochondrial membrane structure and function.

Inclusion of EFA in the diet decreased relative liver mass, whereas DDT caused an

TABLE III

Treatment Means, Factorial Effect Means, and Standard Errors for Various Response Variables[a]

	Treatment mean ± SEM[b]				Factorial effect mean ± SEM			
	Diet							
	1 -DDT-EFA	2 +DDT-EFA	3 -DDT+EFA	4 +DDT+EFA	DDT	EFA	inter-action	standard error
Final body weight (g)[c]	311 ± 11	318 ± 9	465 ± 13	452 ± 13	-3.1[d]	143.8[e]	-9.7[d]	± 9.3
Feed efficiency (g consumed/g final weight)[c]	5.03 ± 0.10	5.05 ± 0.08	3.75 ± 0.05	3.70 ± 0.04	-0.01[d]	-1.31[e]	-0.04[d]	± 0.06
Liver/body ratios (g liver/kg final weight)	37.5 ± 0.8	45.5 ± 0.8	27.3 ± 0.5	36.0 ± 1.1	8.3[e]	-9.9[e]	0.4[d]	± 0.7
Dermatitis score 0 = none 5 = severe	3.9 ± 0.4	4.1 ± 0.5	0.0	0.0	0.1[d]	-4.0[e]	0.0[d]	± 0.3

[a]n = 8 rats/diet.

[b]Standard error of the mean.

[c]Data adjusted for initial body weight by covariance (Ref. 8) SEM from raw data, unadjusted by covariance.

[d]Not statistically significant.

[e]P less than .001.

TABLE IV

Fatty Acid Composition (wt %) of Rat Livers

Fatty acid	Diet 1 -DDT-EFA	Diet 2 +DDT-EFA	Diet 3 -DDT+EFA	Diet 4 +DDT+EFA	Factorial effect mean ± SEM DDT	EFA	Inter-action	Standard errors
12:0	1.6 ± 0.2[a]	0.8 ± 0.1	2.8 ± 0.4	1.7 ± 0.1	-1.0[b]	1.0[b]	-0.2[c]	± 0.2
14:0	2.9 ± 0.2	1.5 ± 0.1	5.7 ± 0.5	4.4 ± 0.2	-1.4[b]	2.8[b]	0.0[c]	± 0.3
16:0	25.0 ± 0.7	21.0 ± 0.6	25.0 ± 0.9	24.0 ± 0.4	-2.9[b]	1.5[d]	0.9[c]	± 0.7
16:1	6.5 ± 0.4	5.3 ± 0.3	3.8 ± 0.2	3.8 ± 0.4	-0.6[c]	-2.1[b]	0.6[c]	± 0.3
18:0	14.0 ± 1.1	18.2 ± 0.8	15.3 ± 0.6	15.5 ± 0.5	2.2[d]	-0.7[c]	-2.0[d]	± 0.8
18:1	30.0 ± 1.0	30.0 ± 1.3	19.4 ± 0.8	20.4 ± 0.6	0.4[c]	-9.9[b]	0.6[c]	± 0.8
18:2	1.4 ± 0.2	2.2 ± 0.2	9.1 ± 0.4	12.8 ± 0.6	2.2[b]	9.2[b]	1.5[b]	± 0.4
20:3ω9	11.3 ± 0.6	13.9 ± 0.8	0.5 ± 0.1	0.3 ± 0.1	1.2[d]	-12.2[b]	-1.4[e]	± 0.5
20:4ω6	5.2 ± 0.3	5.3 ± 0.5	13.4 ± 0.9	13.9 ± 0.6	0.3[c]	8.4[b]	0.2[c]	± 0.4
20:3ω9/20:4ω6	2.2 ± 0.2	2.7 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.2[c]	-2.5[b]	-0.3[c]	± 0.1

[a]Treatment mean ± standard error of the mean.

[b]P less than .001.

[c]Not statistically significant.

[d]P less than .05.

[e]P less than .001.

TABLE V

Fatty Acid Composition (wt %) of Rat Tail Skins

Fatty acid	Diet 1 -DDT-EFA	Diet 2 +DDT-EFA	Diet 3 -DDT+EFA	Diet 4 +DDT+EFA	Factorial effect mean ± SEM DDT	EFA	Interaction	Standard errors
12:0	13.3 ± 0.4[a]	14.1 ± 1.0	21.9 ± 1.0	19.7 ± 0.7	-0.7[b]	7.1[c]	-1.4[d]	± 0.7
14:0	8.9 ± 0.3	8.4 ± 0.3	11.4 ± 0.4	11.0 ± 0.3	-0.5[b]	2.5[c]	-0.1[b]	± 0.3
14:1	1.8 ± 0.2	2.1 ± 0.2	2.2 ± 0.3	2.1 ± 0.1	0.1[b]	0.2[b]	-0.2[b]	± 0.2
16:0	13.4 ± 0.4	13.0 ± 0.4	12.5 ± 0.4	12.3 ± 0.3	-0.3[b]	-0.8[d]	0.1[b]	± 0.4
16:1	12.7 ± 0.6	12.8 ± 0.8	10.3 ± 0.4	10.9 ± 0.5	0.3[b]	-2.1[c]	0.2[b]	± 0.3
18:0	3.2 ± 0.2	3.4 ± 0.2	3.4 ± 0.2	3.2 ± 0.4	-0.1[b]	0.1[b]	-0.3[b]	± 0.2
18:1	37.9 ± 1.2	36.6 ± 0.9	26.2 ± 0.9	28.0 ± 0.9	0.7[b]	-9.7[c]	1.1[b]	± 0.5
18:2	0.9 ± 0.2	1.1 ± 0.1	7.1 ± 0.2	8.4 ± 0.3	0.7[e]	6.7[c]	0.6[d]	± 0.2
20:1	2.6 ± 0.4	2.1 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	-0.3[b]	-1.4[c]	0.2[b]	± 0.2
20:3ω9	1.2 ± 0.2	1.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	-0.1[b]	-1.0[c]	-0.1[b]	± 0.1
20:3ω6	0.7 ± 0.3	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	-0.1[b]	-0.4[e]	0.1[b]	± 0.1
20:4ω6	0.3 ± 0.1	0.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	-0.1[b]	0.7[c]	-0.1[b]	± 0.1
20:3ω9/20:4ω6	7.8 ± 3.0	7.5 ± 1.7	0.2 ± 0.1	0.2 ± 0.1	-0.1[b]	-7.5[c]	0.1[b]	± 1.5

[a]Treatment mean ± standard error of the mean.

[b]Not statistically significant.

[c]P less than .001.

[d]P less than .05.

[e]P less than .01.

increase in relative liver mass, as previously reported (3). The lack of statistically significant interaction between EFA and DDT on the liver/body ratios (P–5.65) suggests that the effects of EFA and DDT on rat liver are independent of one another.

The dermatitis scoring system used (1) requires subjective judgment of the extent and severity of the dermatitis. In spite of this limitation, these scaley eruptions are one of the most notable symptoms of EFA deficiency in rats, and some quantitative estimte of dermatitis is useful. Darsie et al. (9) have reported that 5 ppm *o,p'*-DDT induced an EFAD-like dermatitis in rats fed chow. In our study, DDT did not aggravate the dermatitis of EFAD rats, or induce a dermatitis in the EFA-supplemented animals.

The observed changes in both liver and tail skin fatty acids resulting from EFA supplementation are similar to those that have been reported (1). Depression of the percentages of 16:1, 18:1 and 20:3ω9, and elevation of 18:2 and 20:4ω6 by EFA supplementation, reflects competitive interaction between the 18:2ω6 to 20:4ω6 pathway and the 18:1ω9 to 20:3ω9 pathway (13). EFA supplementation of the diets produced nor al 20:3ω9/20:4ω6 values in both tissues, whereas in tissues from EFAD animal, this ratio was well above the 0.4 level that is indicative of the deficiency state (14).

Tinsley and Lowry (3) reported that DDT increased the 20:3ω9/20:4ω6 ratio in EFAD rat liver total fatty acids. Using this ratio as a biochemical criterion for EFAD severity, they concluded that DDT accentuated EFA deficiency. Darsie et al. (9) have reported that 5 ppm *o,p'*-DDT increased the 20:3ω9/20:4ω6 ratio in hepatic microsomal phospholipids from chow-fed rats from 0.06 to 1.80. They also noted that DDT increased hepatic microsomal phospholipid 16:1, 18:1 and 20:3ω9, decreased 18:2 and 20:4ω6, and reduced 20:3ω6 desaturase activity. The authors concluded that DDT induced an EFAD-like condition in rats fed EFA-adequate diets. In our study, while DDT did evoke complex changes in liver and tail skin fatty acid profiles, the changes were not consistent with an EFA-like deficiency. Although DDT induced a small rise in 20:3ω9 levels in livers of EFAD rats, 20:3ω9/20:4ω6 ratios were not affected by DDT in either tissue studied. Furthermore, DDT actually increased 18:2 levels in both tissues. If 20:3ω6-to-20:4ω6 desaturase inhibition accounted for increased 18:2 in this study, then DDT should have decreased 20:4ω6 levels and this was not observed. Darsie et al. (9) have suggested that DDT induces a functional EFA deficiency in hepatic microsomal phospholipids. If such a deficiency exists, it was not reflected in changes in total liver fatty acids, or in other measures of EFA deficiency considered in our study.

The effects of DDT on tissue fatty acid composition reported here are not readily explained. Tinsley and Lowry (3) suggested that DDT-induced changes in liver fatty acid composition may be related to proliferation of hepatic SER; such proliferation would tend to increase liver 18:2 and 20:4ω6 levels in support of SER membrane biosynthesis. In our study, DDT did increase hepatic 18:2 levels, but the pesticide produced no significant changes in 20:4ω6. If SER proliferation was the primary cause of liver fatty acid changes, then 20:4ω6 levels would presumably have risen along with 18:2.

In the liver, DDT caused significant increases in levels of both 18:2ω6 and 20:3ω9 in EFA-deficient rats. These observations are anomalous since fatty acids of the ω6 series have been shown to inhibit 20:3ω9 biosynthesis (13). One can speculate that DDT blocked ω6-inhibition of 20:3ω9 synthesis.

Within the limits of the experimental design used, it is apparent that while DDT did produce changes in rat liver, including size and fatty acid composition, and in rat tail skin fatty acids, the pesticide did not alter or induce physiological aspects of essential fatty acid deficiency, including the severity of dermatitis, changes in feed efficiency and changes in growth and final body weight. However, while DDT may not have exacerbated EFA deficiency, and while an explanation of this biochemical effects remains elusive, the pesticide's effect on liver size and on the fatty acid composition of both liver and tail skin make it clear that chronic DDT ingestion does have an unexplained effect on lipid metabolism in the rat.

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Characterization of Plasma Lipoproteins in Swine with Different Propensities for Obesity

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ABSTRACT

Yorkshire (lean) and Ossabaw (obese) swine ca. one year of age were used to characterize the quantity and composition of plasma lipoproteins in animals with markedly different adiposity. While lean swine weighed more (175 vs 88 kg for obese), they had less backfat than obese swine (2.64 vs 5.97 cm; $P < 0.05$). Fasting plasma triacylglycerol (Tg) and cholesterol (CH) levels were elevated in obese swine. Swine plasma lipoproteins were fractionated into very low density lipoprotein (VLDL; $d < 1.006$), low density lipoprotein$_1$ (LDL_1; $d = 1.019$-1.063), low density lipoprotein$_2$ (LDL_2; $d = 1.063$-1.09), and high density lipoprotein (HDL; $d = 1.09$-1.21) by density ultracentrifugation. Obese VLDL-Tg, CH and protein (Pr) were elevated more than 2-fold. VLDL from obese swine were 2-fold larger than VLDL from lean swine. No alterations in LDL_1 or LDL_2 composition were observed. HDL-Tg, CH, Pr and phospholipid levels were significantly higher in obese swine. Plasma and VLDL-Tg levels were highly correlated with backfat thickness ($r = 0.67$ and $r = 0.73$, respectively). There was a positive correlation between adiposity and HDL-CH as well as VLDL-Tg and HDL-CH. These data indicate that (a) there are marked alterations in swine plasma lipoprotein composition between lean and obese swine; (b) that swine plasma lipoprotein levels may be useful parameters in estimating body composition; and (c) that HDL-CH is positively correlated with adiposity in swine.

INTRODUCTION

The plasma lipoprotein profile for miniature and lean swine is similar to that reported for man. Swine plasma contains 3 classes of lipoproteins: very low density lipoprotein (VLDL), 2 classes of low density lipoprotein (LDL_1 and LDL_2) and high density lipoprotein (HDL) (1). In addition, the apoprotein composition of swine HDL is similar to that of the human (2). There have been no studies reported to date characterizing the lipoprotein profiles of swine with different propensities for obesity. Studies in man have demonstrated that obese subjects have higher circulating triacylglycerol (Tg) levels (3) and lower HDL-cholesterol levels (4,5) than normal subjects. The elevation in plasma triacylglycerol levels is primarily attributable to an elevation of VLDL triacylglycerol (5).

In swine, the adipose tissue mass can undergo prolific expansion during growth. At birth, the adipose tissue mass may comprise 1-2% of body weight whereas by 6 months of age this may be as high as 40-50% (6). In comparison with other species, relatively little is known about the role of plasma lipoproteins in the accretion of adipose tissue during animal growth. Work recently conducted in our laboratory has demonstrated a progressive decrease in adipocyte fatty acid synthesis during the growth of adipose tissue (7). Palmitate esterification increases during this same interval, suggesting that adipose tissue expansion becomes more dependent on fatty acids derived from plasma for Tg synthesis within the adipocyte (7). To assess the involvement of plasma lipoprotein Tg as a source of fatty acids for intracellular esterification, it is necessary to determine whether lipoprotein profiles are different between lean and obese swine prior to conducting studies to ascertain whether these differences may be involved in the development of obesity in swine. Data reported herein indicate that marked differences exist in VLDL and HDL composition between lean and obese swine.

MATERIALS AND METHODS

A breed of swine (Ossabaw) with a marked propensity for obesity has been extensively studied in our lab (8-10). This obesity, genetic in nature, is not associated with a marked hyperphagia (11) and displays many of the endocrine and metabolic phenomena associated with obesity in man and other animal models (12). In particular, nonfasting insulin levels are elevated (12), plasma growth hormone levels are suppressed (12) and obese swine have higher rates of lipogenesis and gluconeogenesis (9).

Female swine ca. 10-12 months of age were used for these experiments. Lean Yorkshire swine (N=16) weighed 175 ± 7 kg and obese Ossabaw swine (N=22) weighed 88 ± 2 kg. Yorkshire swine were used to depict animals

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which do not have a propensity for obesity. One indirect measurement routinely used to assess adipose tissue mass in swine is average backfat thickness. This measurement is highly correlated with body fat content (13) and is the average thickness of adipose tissue from the epidermis to the fascia covering the skeletal muscle dorsal to the first rib, last rib and last lumbar vertebrae. Therefore, while lean swine weighed more, they had significantly less backfat than obese swine (2.64 ± .15 cm vs 5.97 ± .20 cm, $P < 0.05$). Some of the swine were pregnant; however, no statistical differences in VLDL, LDL_1, LDL_2, or HDL composition were found between nonpregnant and pregnant animals within a breed. Furthermore, there was no breed x group interaction.

Blood samples were collected (0900 hr) after an overnight fast via exsanguination. Blood was collected into centrifuge tubes containing EDTA (1 mg/ml blood). Plasma was prepared by centrifugation for 15 min at 2500 rpm at 4 C. Sodium azide was added to the plasma to a final concentration of 0.02%. Typically, separation of lipoprotein fractions was begun immediately after collection of plasma. However, some samples were stored overnight at 4 C before commencement of lipoprotein fractionation. Plasma was fractionated by ultracentrifugation using established procedures (14). Previous results indicate that swine plasma has 2 LDL-like particles. Thus, VLDL, LDL_1, LDL_2 and HDL were isolated from the plasma of each animal at $d < 1.006$, 1.019-1.063, 1.063-1.09 and 1.09-1.21, respectively (15). Density adjustments were made by addition of solid KBr. Density measurements were made with pycnometers. Lipoprotein fractions were obtained by ultracentrifugation in a Beckman L2-65B centrifuge using a Beckman SW-40 rotor at 15 C. VLDL were isolated by centrifugation for 1.1×10^8 g min, LDL_1 and LDL_2 for 1.4×10^8 g min, and HDL for 2.7×10^8 g min. HDL were collected and subjected to recentrifugation (2.7×10^8 g min) to remove traces of albumin. Lipoprotein purity of all fractions was determined with agarose gel electrophoresis (16).

Plasma lipoprotein composition was determined for each animal by measuring triacylglycerol (17,18), cholesterol (19), phospholipid (20) and protein (21). Each parameter was assayed in triplicate. Deoxycholic acid (200 µl of 5% solution) was used to remove the turbidity associated with lipid for all protein assays (22). Apoprotein B content was measured following precipitation with tetramethylurea (23). Each plasma sample was spun at d = 1.006-1.019 to determine if an intermediate density lipoprotein (IDL) was present. No IDL was observed in any of the plasma samples from lean or obese swine with agarose gel electrophoresis nor was there any discernable triacylglycerol, cholesterol, phospholipid or protein present in this density fraction.

Statistical comparisons were conducted by Analysis of Variance (ANOVA). Treatment differences for all measured parameters between lean (N=16) and obese swine (N=22) were determined using an F-test. Linear regression and correlation coefficients were calculated for selected parameters (24). Statistical measurements were conducted to discern if the slope of the regression line for lean and obese swine differed from each other for each comparison. Since the slopes were not different, linear regression analyses were conducted for both groups together.

RESULTS

Fasting plasma Tg was significantly elevated in obese swine (Table I). Although statistically significant, plasma cholesterol levels were only modestly elevated in obese swine (77 vs 85 mg% for lean and obese swine, $P<0.05$). Unlike all other measurements, there were statistical differences for plasma cholesterol values from pregnant and nonpregnant obese swine. However, the variation attributable to these differences was appropriately partitioned by the ANOVA so that breed effects (i.e., comparisons of lean vs obese) could be tested. From the ANOVA, a confidence interval of the difference between average plasma cholesterol values for lean and obese swine was computed. The resulting 95% confidence interval indicated that the difference between obese (μ_1) and lean (μ_2) swine was significantly different ($0.05<\mu_1-\mu_2<15.6$). Thus, from 2 criteria, plasma cholesterol values were judged to be significantly elevated; however, it is important to note that the difference was not appreciable. The content of Tg, cholesterol and protein in $d < 1.006$ was greater in plasma from obese swine (Table I). Apoprotein B levels as a percentage of total VLDL protein were similar for lean and obese swine (7.6 vs 7.0%, respectively). VLDL-Tg levels in particular were elevated more than 2-fold. The composition of LDL_1 differed in lean and obese swine; LDL_1 isolated from lean swine contained more Tg and PL. However, the composition of LDL_2 in lean and obese swine was similar. All parameters measured in the HDL fraction (d = 1.09-1.21) were higher in obese swine (Table I).

About 80% of the plasma Tg was associated with VLDL in the lean whereas 90% of plasma

TABLE I

Composition of Plasma Lipids and Lipoprotein Fractions from Lean and Obese Swine[a]

Plasma triacylglycerol	Lean	Obese
	52 ± 3[f]	86 ± 6[g]

	VLDL[b]		LDL_1[c]		LDL_2[d]		HDL[e]	
	Lean	Obese	Lean	Obese	Lean	Obese	Lean	Obese
Triacylglycerol	28.0 ± 2.8[f]	65.3 ± 6.1[g]	3.8 ± 0.4[f]	3.0 ± 0.2[g]	2.8 ± 0.4[f]	2.3 ± 0.3[f]	0.9 ± 0.2[f]	1.6 ± 0.2[g]
Cholesterol	2.9 ± 0.3[f]	5.2 ± 0.5[g]	25.9 ± 2.8[f]	20.3 ± 2.3[f]	16.0 ± 1.8[f]	16.0 ± 1.4[f]	7.0 ± 0.5[f]	11.1 ± 0.9[g]
Protein	4.4 ± 0.4[f]	7.8 ± 0.7[g]	21.8 ± 2.9[f]	17.8 ± 2.0[f]	18.0 ± 2.0[f]	17.0 ± 1.6[f]	17.8 ± 2.5[f]	31.4 ± 2.4[g]
Phospholipid	13.3 ± 1.0[f]	15.5 ± 1.4[f]	30.9 ± 2.9[f]	20.3 ± 1.8[g]	13.2 ± 2.3[f]	13.7 ± 1.4[f]	12.7 ± 1.3[f]	22.5 ± 1.6[g]

[a]Lean swine (n=16) had 2.64 ± 0.15 cm of backfat. Obese swine (n=22) had 6.97 ± 0.2 cm of backfat. Values for all measurements are expressed as mg % (mean ± SEM).

[b]Very low density lipoproteins (VLDL); $d < 1.006$.

[c]Low density lipoproteins (LDL_1); $d > 1.019, < 1.063$.

[d]Low density lipoproteins (LDL_2); $d > 1.063, < 1.09$.

[e]High density lipoproteins (HDL); $d > 1.09, < 1.21$.

[f,g]Plasma triacylglycerol values with a different superscript indicate that the means are significantly ($P < 0.01$) different for lean and obese swine. Different superscripts for a respective lipoprotein constituent within a lipoprotein fraction indicate that the means are significantly different ($P < 0.05$) between lean and obese swine.

Tg was associated with VLDL in the obese swine. Although cholesterol was transported principally by the 2 LDL fractions, there were differences in the relative proportion of cholesterol present. More than 80% of plasma cholesterol was transported by LDL_1 and LDL_2 in lean swine. LDL_1 and LDL_2 from obese swine accounted for less than 70% of the cholesterol in the plasma.

There was a marked shift in the proportion of phospholipid, protein and cholesterol transported by HDL between the 2 groups. A greater proportion of lipoprotein phospholipid, protein and cholesterol was transported by HDL from obese swine. The proportion of plasma cholesterol transported in the HDL fraction was ca. 14 and 21% for lean and obese swine, respectively. This shift in prominence by lipoproteins of d = 1.09-1.21 between lean and obese swine was not noted for Tg, which comprised a very modest proportion of HDL (ca. 3% of total lipoprotein Tg was associated with HDL of lean and obese swine).

The relative proportions of plasma Tg and cholesterol transported by the respective lipoprotein fractions were based on the quantity of Tg and cholesterol recovered in all the lipoprotein fractions. Recovery of total cholesterol in the density fractions compared to plasma values was ca. 65% for both lean and obese swine. Recovery of total Tg in the density fractions was 82 and 68% of the measured plasma values for obese and lean swine, respectively. Relative differences in recovery of Tg or cholesterol in the density fractions vs plasma levels was presumably because of losses during subsequent ultracentrifugation and washing of higher density fractions (25). The relative difference in recovery of Tg in the density fractions between lean and obese swine likely reflects the fact that a greater proportion of plasma Tg is transported in the higher density fractions in lean swine. Thus, a proportionally greater loss of Tg in density fractions subsequent to VLDL would be expected.

To clarify the association of obesity with selected lipoprotein parameters, linear regression analyses were conducted. Plasma triacylglycerol levels increased with increasing backfat thickness (Fig. 1). The proportion of variation that was attributable to the independent variable, backfat thickness, was 45% (r^2 = .45). The correlation coefficient and coefficient of determination between VLDL-Tg and backfat thickness was greater (Fig. 2) than for plasma triacylgycerol backfat thickness. Fifty-three percent of the variation in VLDL-Tg levels was associated with changes in backfat thickness. Our results indicate that a positive linear

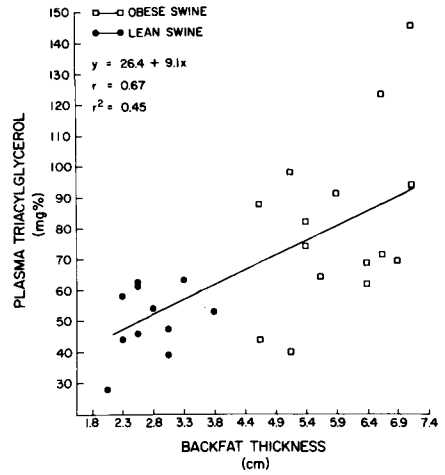

FIG. 1. Linear regression analysis of plasma triacylglycerol and swine adiposity.

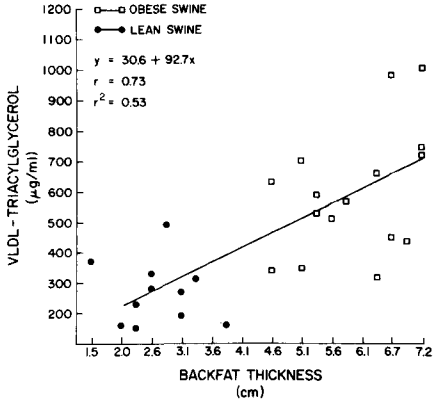

FIG. 2. Linear regression analysis of VLDL-triacylglycerol and swine adiposity.

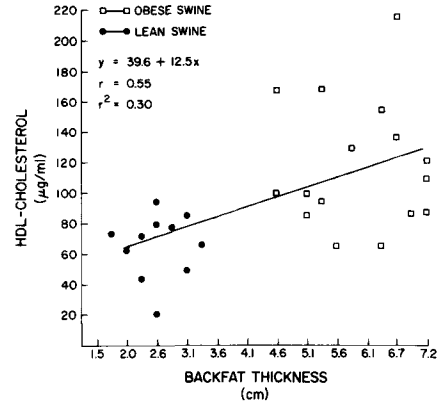

FIG. 3. Linear regression analysis of HDL-cholesterol and swine adiposity.

relationship exists between swine HDL-cholesterol levels and obesity (Fig. 3). From data presented in Figures 2 and 3, it is evident that a positive relationship exists between HDL-cholesterol and VLDL-Tg.

DISCUSSION

Studies to elucidate the role of lipoproteins in the accretion of swine adipose tissue are contingent upon the characterization of plasma lipoproteins from lean and obese swine. In particular, a determination of the differences in the lipoprotein profile between lean and obese swine is necessary to contribute to our understanding of adipocyte growth as influenced by lipoprotein composition. Recent investigations have shown both binding and internalization of various lipoproteins to isolated rat adipocytes (26). What occurs subsequently, however, in terms of fatty acid or triacylglycerol synthesis and the accretion of lipid in the adipocyte has not been elucidated. We have documented that obese swine have elevated plasma Tg and cholesterol levels compared with lean swine. Furthermore, these differences are manifested as marked alterations in VLDL and HDL composition. It is unlikely that these observations result from differences in feed intake since obese swine are not markedly hyperphagic (11); furthermore, in this experiment, lean and obese swine were fed similar amounts of feed/kg body weight.

The diet fed to the swine is analogous to other high carbohydrate diets reported to induce hypertriglyceridemia in the rat (27,28) as well as man (29). It is unlikely that the amount of dietary carbohydrate in our diet had an appreciable effect on VLDL-Tg levels. Two studies with miniature pigs have indicated that plasma triacylglycerol and VLDL-Tg levels are unaltered by decreasing the carbohydrate and increasing the dietary lipid content of the diet (30,31). However, feeding diets containing 1.5-3% cholesterol in addition to 15-20% lard or tallow (weight basis) causes marked increases in plasma cholesterol (30,31). This increase in plasma cholesterol is manifested largely as an α-migrating lipoprotein referred to as HDL_c (cholesterol induced) in d = 1.02-1.087 (31). Whether the addition of dietary lipid or cholesterol will induce similar alterations in the composition and type of lipoproteins and apoprotein content in obese swine is unknown.

Although apoprotein B levels as percentage of VLDL protein were not different for lean and obese swine, the absolute quantity of apoprotein B was higher in obese swine because of the elevation in VLDL protein levels. Since apoprotein B levels on an absolute basis were higher in obese swine, this infers that part of the difference in VLDL-Tg is because of differences in particle number. Other evidence such as the ratio of phospholipid (PL) to Tg as an approximation of particle size (32,33) supports the contention that a difference also exists in particle size. A relative decrease in PL:Tg ratio is indicative of an increase in particle size; VLDL PL:Tg was .48 for lean swine and .24 for obese swine, which suggests that VLDL particle size is different between lean and obese swine.

It is unclear whether this increase in VLDL-Tg reflects a perturbation in hepatic production, extrahepatic removal or a combination of both. In man, hypertriglyceridemia is associated with several factors. Increased plasma FFA levels increase hepatic VLDL-Tg production in obese subjects (34,35). It has been shown that plasma insulin is elevated in obese subjects and this is highly correlated with elevations in VLDL-Tg levels (36,37). Wangsness et al. (12) have reported elevated plasma insulin levels in obese swine. Based on our results and those of Wangsness et al. (12), the increased VLDL-Tg in obese swine may reflect an augmented hepatic triglyceride production which is secondary to elevated insulin levels. This contention is further supported by evidence that insulin can augment hepatic lipoprotein synthesis and secretion in man (36). Although lipoprotein removal rates were not measured, data reported suggest that removal of VLDL by extrahepatic tissue is not decreased in obese swine; rather it may be increased. Lee and Kauffman (38) measured lipoprotein lipase (LPL) activity in swine adipose tissue during growth to 6 months of age and observed that LPL activity/cell progressively increased during adipose tissue growth. We have found that adipocyte palmitate esterification increases markedly with increasing adiposity in swine at a time when glucose conversion to triacylglycerol decreases (7). It has not been resolved if this increase in esterification is the result of an enhanced use of plasma FFA or reflects elevated LPL activity, thereby increasing the availability of fatty acids for intracellular esterification. Nonetheless, these findings, in conjunction with the report by Reaven et al. (27) documenting that rat adipose tissue LPL was slightly higher in hypertriglyceridemic rats, indicate that removal of VLDL-Tg may not be impeded in obese swine. Furthermore, based on the limited LPL data at hand, the obese swine may actually have an accelerated rate of VLDL-Tg removal by adipose tissue.

Our results are the first to indicate that LDL_1 and LDL_2 composition of plasma from

obese swine is similar to lean swine. The composition of plasma LDL_1 and LDL_2 from lean and obese swine agrees with data previously reported demonstrating that the major cholesterol transport lipoproteins in swine are low density lipoproteins (31).

Of considerable importance is the finding that a positive correlation exists between adipose tissue mass and HDL cholesterol in swine with markedly different propensities for obesity. The relationship between adiposity and HDL cholesterol in man is not yet clear. While numerous investigators have reported an inverse correlation between HDL cholesterol and adiposity, confounding factors such as diet, exercise and alcohol consumption make interpretation of the data difficult (39). However, in most instances, 10% or less of the variation in HDL cholesterol is attributable to differences in adiposity (39).

Investigations of the lipoprotein profile of obese Zucker rats have demonstrated a significant increase in all lipoprotein classes concommitant with a massive hyperlipidemia (40). Plasma cholesterol and triglyceride levels are markedly elevated in obese rats. Although HDL composition is similar between lean and obese rats, HDL levels are 2-fold higher in obese animals (40). The HDL moiety of genetically obese mice is cholesterol-enriched; however, plasma triglyceride levels are normal (41).

Our data do not agree with investigations conducted on humans which have reported an inverse correlation between plasma Tg and HDL cholesterol (42) and VLDL-Tg and HDL cholesterol (43). Moreover, our results are different from investigations conducted in other genetically obese species (40,41). Therefore, relationships and correlations among plasma Tg and VLDL-Tg, adiposity and HDL cholesterol are unclear at this time. Whether changes in HDL-cholesterol between lean and obese swine can be manifested by diet, weight loss or exercise is worthy of investigation.

The correlation between VLDL-Tg and adiposity suggests that measurements of plasma VLDL-Tg levels may be useful in predicting adipose tissue mass in swine. In animal agriculture, there is a continuing need for simple techniques to assess body composition in the live animal. Other methods are available for estimation of composition and include measurements of subcutaneous adipose thickness (13), total body water (44), body density (45) and body potassium content (46). However, measurements of body density, potassium and body water are rather detailed procedures to use for meat animals. Based on previous work indicating that backfat thickness is highly correlated with obesity (13), the results of this experiment suggest that inclusion of VLDL-Tg levels as a variable in a multiple regression equation with backfat thickness may increase the accuracy of predicting body adipose tissue mass.

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Studies on Phospholipase Activities in *Neurospora crassa* mycelia

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ABSTRACT

Phospholipase A activity has been detected in mycelial homogenate of *Neurospora crassa*. A submycelial fraction, obtained by differential centrifugation containing the highest specific activity of phospholipase A has been shown to contain ca. 66% phospholipase A_1 and 34% phospholipase A_2 activity along with lysophospholipase and degergent-stimulated phospholipase D activity. Phospholipase A activity bound to *N. crassa* mycelia also has been observed.

INTRODUCTION

A large number of studies have been carried out on the chemical and biochemical aspects of the deacylation of phosphoglycerides by phospholipase A_1 (EC 3.1.1.32) and phospholipase A_2 (EC (3.1.1.4) of animal tissues. Activities of these 2 enzymes have also been described in different microorganisms. Phospholipase A_1 apparently is the predominant lipolytic enzyme of bacteria (1). Comparatively few studies have been carried out on phospholipase A activity in fungi (2-5).

In *Neurospora crassa*, lysophospholipase (EC 3.1.1.5) activity has been previously described in its cell-wall fragments (6). In this paper, the presence of phospholipase A_1 and phospholipase A_2 in the mycelia of *N. crassa* is reported along with lysophospholipase and a detergent-stimulated phospholipase D (EC 3.1.4.4) activity. A 20,000 × g pellet fraction, containing the highest specific activity (sp act) of phospholipase A, has been used for detailed study of some enzyme properties.

MATERIALS AND METHODS

Growth of *N. crassa*

Stock culture of *N. crassa* ITCCF 1701 (wild, mating allele A) was obtained from the Indian Agricultural Research Institute (India). The culture medium used for growth was based on Dox and Thom's modification of Czapek's medium (7) and consisted of glucose (5%), $NaNO_3$ (0.2%), KH_2PO_4 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.05%), KCl (0.05%), $FeSO_4 \cdot 7H_2O$ (0.001%), yeast extract (0.05%) and malt extract (0.05%), all w/v. Yeast and malt extracts were obtained from Centron Research Laboratories, India. Other chemicals used were GR grade, supplied by E. Merck, Germany.

N. crassa conidia from stock cultures were transferred to Erlenmeyer flasks (500 ml) containing 100 ml of the sterile culture medium already described solidified with 2% Bacto Agar (Difco Laboratories, Detroit, MI) and incubated at 34 C for 6-7 days, and a conidial suspension was prepared with the mature conidia (8).

For obtaining vegetative mycelia of *N. crassa*, Erlenmeyer flasks (250 ml), each containing 50 ml of the sterile liquid culture medium, were inoculated with 10^7 fresh conidia and incubated at 34 C without shaking. After 5-7 days, the mycelia were harvested by filtration and washed several times with distilled water. Mycelial growth was measured by determination of mycelial dry weight (9).

Preparation of Mycelial Homogenate

Harvested mycelia were blotted to remove excess moisture, disrupted by grinding with twice its weight of precooled neutral alumina (Sarabhai M. Chem., India) in a mortar and pestle at 2-4 C using 0.25 M sucrose and centrifuged 3 times at 1,500 × g for 15 min at 2-4 C. The final supernatant, viz. mycelial homogenate, was used.

Differential Centrifugation of Mycelial Homogenate

The mycelial homogenate was successively centrifuged at 12,000 × g for 30 min; 20,000 × g for 30 min; and 110,000 × g for 60 min at 2-4 C, 3 times in each case. The ultimate supernatant (soluble supernatant) and the pellets obtained in each case were suspended in 0.25 M. sucrose and assayed for enzyme activity.

Protein Estimation

Protein was estimated by the Biuret method (10).

Assay of Phospholipase A Activity

A: *Hydrolysis of aqueous suspension of egg yolk phosphoglycerides.* The substrate-egg yolk suspension was prepared in 0.9% NaCl as described by Marinetti (11). In our assay

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system, the initial absorbance at 925 nm was 0.4 instead of 0.6. This change had no effect on the nature of the enzyme activity-time curve. Incubation conditions were essentially the same as described by Marinetti (11). The assay mixture consisted of 1 ml substrate, 0.5 ml enzyme preparation and 4.5 ml 0.9% NaCl. Unless otherwise mentioned, enzyme assays were carried out at 40 C for 10 min. One turbidimetric unit of phospholipase A activity was taken to be equal to the change of absorbance of 0.1 at 925 nm/min. Specific activity (sp act) was expressed as units/mg protein. For quantitative determination of lysophosphoglycerides formed, the reaction was stopped with 5 ml of chloroform/methanol (2:1, v/v) and lysoglycerophosphatides were identified, separated by thin layer chromatography (TLC) and estimated as described in assay method B.

B: *Hydrolysis of phosphoglycerides in mixtures of aqueous buffer and ether or methanolic ether.* Substrate phosphatidylcholine (PC) L-α-(β-γ-dipalmitoyl) and phosphatidylethanolamine (PE) L-α-(β-γ-dipalmitoyl), both of synthetic A grade, were obtained from Calbiochem, LaJolla, CA. Lecithin-(1-stearyl-2-oleoyl) was obtained from Applied Science Laboratories, Inc., State College, PA. Crude egg PC and egg PE were isolated from hen egg yolk (12) and purified by preparative TLC (Silica Gel H [E. Merck, Germany] 0.35 mm/20 × 20 cm, solvent system-chloroform/methanol/water [65:25:4, v/v/v]).

Incubation conditions were modified from Kates (13). The assay mixture consisted of 0.5 ml of buffer solution (0.2 M boric acid-borax buffer, pH 7.2 for mycelial homogenate or 0.2 M acetic acid-sodium acetate buffer, pH 5.5 for 20,000 × g pellet), 0.1 ml 1 M $CaCl_2$ solution, 0.5 ml enzyme preparation and 4.08 μmol glycerophosphatide substrate dissolved in 1 ml organic solvent (diethyl ether or diethyl ether/methanol [95:5 or 90:10, v/v]). Assays were performed in 10-ml screw-capped tubes and incubated at 40 C for 3 hr under continuous shaking. The reaction was stopped with 5 ml of chloroform/methanol (2:1, v/v) and lysoglycerophosphatides were identified and estimated by TLC as described next. In the control, the enzyme was added after addition of the solvent mixture. The separated components were identified by spraying the developed TLC plates (Silica Gel H, 0.35 mm/20 × 20 cm, solvent system, chloroform/methanol/water [65:25:4, v/v/v]), with ninhydrin reagent (14) for PE and lysophosphatidylethanolamine (LPE), Dragendorff reagent (15) for PC and lysophosphatidylcholine (LPC) and molybdenum blue reagent (16) for PC, LPC, and PE, LPE. For quantitative measurement of lysophosphoglycerides formed, the compounds were visualized with iodine vapor (17-19), the necessary bands were scraped off and extracted 3 times with 5 ml of chloroform/methanol/water (65:25:4, v/v/v). Suitable aliquots of the combined extracts were taken for phosphorus estimation. Organic phosphate was first converted to inorganic phosphate (20) and the inorganic phosphate estimated (21). Blanks were measured following a similar procedure by removal of an equal area of adsorbent from the plate.

Authentic phosphoglycerides used in TLC were a generous gift from Professor S. Numa, Kyoto University, Kyoto, Japan.

C: *Assay of phospholipase A activity bound to fungal mycelia.* Incubation conditions were essentially the same as described by Blain et al. (5). The assay mixture consisted of 4.08 μmol dipalmitoyl PC dissolved in 1 ml diethyl ether/methanol (95:5, v/v), 80-100 mg defatted mycelial powder prepared by the method of Blain et al. (5) and 80 μl water. Assays were performed in 10-ml screw-capped tubes at 37 C for 3 hr under continuous shaking. The substrate was omitted in the control. The rest of the method was similar to that in assay method B.

Gas Liquid Chromatography (GLC)

Methyl esters of fatty acids in LPC were prepared according to Luddy et al. (22). These were analyzed by gas liquid chromatography (GLC) in a F&M model 700 R Gas Chromatogram equipped with a flame ionization detector and a 15% diethylene glycol succinate polyester supported on 60-80 mesh Gas-Chrom P column (Applied Science Laboratories Inc.) at 170 C.

Analysis of Water Soluble Products of Hydrolysis

After the chosen period of incubation, 5 ml chloroform/methanol (2:1, v/v) was added to the reaction mixture in assay method (B) followed by 4 ml water. Layers were allowed to separate. The aqueous phase was collected, concentrated in a stream of nitrogen and analyzed by ascending paper chromatography Whatman No. 1 paper, developing solvent system methanol/98% formic acid/water (80:13:7, v/v/v) for analysis of water soluble glycerophosphoryl bases. Developed chromatograms were visualized by spraying with molybdenum blue reagent (16) for both glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE), ninhydrin reagent (14) for GPE and Dragendorff reagent (15) for

GPC. Standard L-α-GPC and L-α-GPE were obtained from Sigma Chemical Co., St. Louis, MO.

General Comments

Any result expressed here is a mean value of 5 separate determinants. Standard error is ± 5%.

RESULTS

Phospholipase A Activity in the Mycelial Homogenate

The ability of the mycelial homogenate obtained from 5-day-old *N. crassa* mycelia (16 mg protein/ml) as an enzyme source to hydrolyze solutions of egg PC, dipalmitoyl PC, egg PE and dipalmitoyl PE, respectively, in ether; ether/methanol (95:5, v/v); ether; and ether/methanol (90:10, v/v) was examined by assay method B. Separate control experiments in which either the substrate or the enzyme was omitted showed the absence of any nonenzymatic formation of LPC or LPE. Under these conditions, 4.08 μmol dipalmitoyl PC was hydrolyzed to 2.2 μmol LPC in 3 hr, corresponding to 53.9% hydrolysis.

Clearing Action on the Turbidity of Egg Yolk Suspension

Mycelial homogenate obtained from 5-day-old *N. crassa* mycelia (1 mg protein/ml) was tested for its clearing action on the turbidity of a suspension of hen egg yolk in 0.9% NaCl (11) by assay method A. Considerable phospholipase A activity is present in the mycelial homogenate. Both nonenzymatic decrease of absorbance and formation of LPC and LPE were absent. An initial lag period observed (11) in the clearing of egg yolk suspension by some phospholipase A_2 also was absent. The clearing of the turbidity of egg yolk suspension by the mycelial homogenate was linear with time, at least up to 10 min (Fig. 1). The effect of different amounts of protein in the mycelial homogenate on the phospholipase A activity was determined by the turbidimetric method (Fig. 2). Enzyme activity increased linearly with increased amounts of mycelial protein in the incubation mixture, up to 500 μg protein. A good correlation was obtained between enzyme activity in terms of turbidimetric assay units and the net amount of LPC (and LPE) formed (Fig. 3). Figure 3 shows that

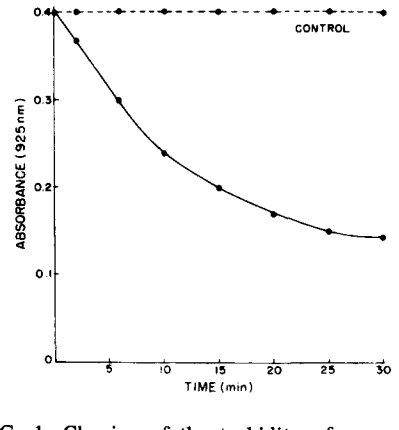

FIG. 1. Clearing of the turbidity of an egg yolk suspension with time by the mycelial homogenate from *N. crassa*. The assay mixture consisted of 1 ml egg yolk suspension, 0.5 ml enzyme preparation (500 μg protein) and 4.5 ml 0.9% NaCl. Enzyme was omitted in the control which consisted of 1 ml egg yolk suspension and 5 ml 0.9% NaCl. Incubation was carried out at 41 C. Absorbance was monitored at 925 nm.

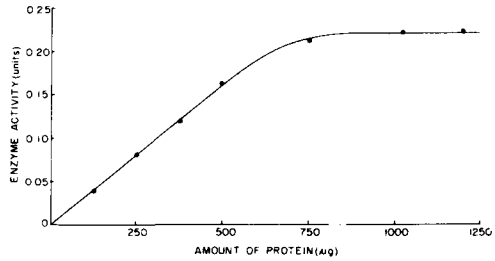

FIG. 2. The relationship between phospholipase A activity and the amount of protein of the mycelial homogenate from *N. crassa*. The assay mixture consisted of 1 ml egg yolk suspension, 0.5 ml enzyme preparation and 4.5 ml 0.9% NaCl. Incubation was carried out at 41 C for 10 min.

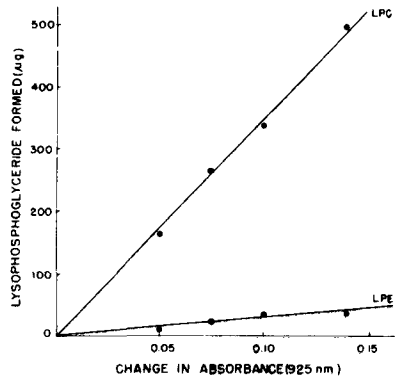

FIG. 3. Relationship between change in absorbance at 925 nm and LPC or LPE formed in the egg yolk clearing method. The assay mixture consisted of 1 ml egg yolk suspension, 0.5 ml enzyme preparation (150 μg or 225 μg or 300 μg or 400 μg protein in different experiments) and 4.5 ml 0.9% NaCl. The reaction was carried out at 41 C for 10 min.

1 turbidimetric unit of phospholipase A activity is equivalent to formation of 345 μg LPC/min.

Variation of Phospholipase A Activity in Mycelial Homogenate with Growth

Variation of phospholipase A activity in the mycelial homogenate with age of the mycelium was determined (Fig. 4) by assay method A. Highest sp act of phospholipase A was observed during the late stationary phase of the asexual growth of the fungal mycelium at the interval of 132-155 hr. In all subsequent studies, unless otherwise stated, mycelia were harvested during this time interval.

Sucrose (0.25 M) or NaCl (0.9%) As Disruption Medium

Phospholipase A activity was determined by assay method A using mycelial homogenate (1 mg protein/ml) in 0.25 M sucrose or 0.9% NaCl. The average sp act of phospholipase A was 62.5% higher in mycelia disrupted in 0.9% NaCl than that in 0.25 M sucrose.

Differential Centrifugation Studies

Six-day-old *N. crassa* mycelia were disrupted in 0.25 M sucrose and the mycelial homogenate was subjected to differential centrifugation. The total and the mean sp act of phospholipase A in the fractions thus obtained were determined (Table I). Different pellets obtained were suspended in 0.25 M sucrose and preparations containing 100 μg protein/ml used for determination of phospholipase A activity by assay method A.

The 20,000 × g pellet contained the highest sp act of phospholipase A. Also, the sp act of phospholipase A in the 20,000 × g pellet was 64% higher when suspended in 0.9% NaCl than that in 0.25 M sucrose. In all subsequent studies, mycelia were disrupted in 0.25 M sucrose and the 20,000 × g pellet suspended in 0.9% NaCl was used.

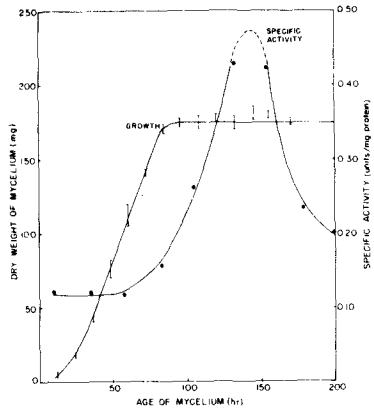

FIG. 4. Variation of phospholipase A activity in *N. crassa* mycelial homogenate with asexual growth of the fungus. The vertical lines show the range of values obtained for dry mycelial weight/50 ml MgZ medium. The line (●—●) shows the specific activity at different phases of growth. The assay mixture consisted of 1 ml egg yolk suspension, 0.5 ml enzyme preparation (500 μg protein) and 4.5 ml 0.9% NaCl. Incubation was carried out at 41 C for 10 min.

Properties of Phospholipase A in the 20,000 × g Pellet

The optimal temperature of phospholipase A activity was 40 C (Fig. 5). Upon storage at -20 C for 17 hr, there was a 38.1% loss of phospholipase A activity. Lyophilization had no adverse effect on phospholipase A activity. The activity was unaffected during storage at 0 C for at least 7 days. After heating the enzyme preparation to 60 C for 5 min, the sp act of the enzyme was doubled. Exposure to 80 C for 5 min caused a 50% decrease in sp act, whereas at a higher temperature (5 min at 100 C), the enzyme activity was almost completely abolished. Presonication of the enzyme preparation for 10 min in an Ultrasonic Cleaner (Laboratory Supplies Co. Inc., Hicksville, NY) had an activating effect (70%) on the phospho-

TABLE I

Total and Mean Specific Activities of Phospholipase A in Fractions Obtained by Differential Centrifugation

Fractions	Total activity	Mean specific activity (units/mg protein)
1,500 × g supernatant	122.11	0.43
12,000 × g pellet	30.12	0.50
20,000 × g pellet	88.56	3.20
110,000 × g pellet	8.31	0.45
110,000 × g supernatant	238.92	1.40

lipase A activity. All properties were determined using assay method A.

In Table II, the influence of several activators and inhibitors on the phospholipase A activity is summarized. TLC analysis (Silica Gel H, 0.35 mm/20 × 20 cm, solvent systems-chloroform/methanol/water [65:25:4, v/v/v] and diisobutyl ketone/glacial acetic acid/water [40:25:5, v/v/v] in separate experiments) of lipids isolated from assay mixture containing detergents showed the formation of considerable amounts of phosphatidic acid (PA). Control experiments showed the absence of formation of PA both nonenzymatically in the presence of detergents and enzymatically in the absence of detergents. This suggests activation of phospholipase D activity in the presence of detergents. The optimal pH of phospholipase A activity was ca. 5.5 (Fig. 6).

Effect of Substrate Concentration

The effect of substrate concentration (s) on the initial rate of hydrolysis (v) of L-α-dipalmitoyl PC (synthetic) by phospholipase A in the 20,000 × g pellet was studied by assay method B and the apparent K_m (5) was determined to be ca. 4.4×10^{-3} M (Fig. 7).

Type of Phospholipase A Activity

In studies on the type of phospholipase A activity in the 20,000 × g pellet (1 mg protein/ml), enzyme reactions were carried out as in assay method B. LPC fractions were separated by TLC and analyzed by GLC for fatty acid composition.

In initial experiments, it was confirmed that the fatty acids in LPC formed upon enzymic hydrolysis of PC by the 20,000 × g pellet originated from the substrate PC only and not from any endogenous source since 16:0 was the only fatty acid in LPC obtained from dipalmitoyl PC.

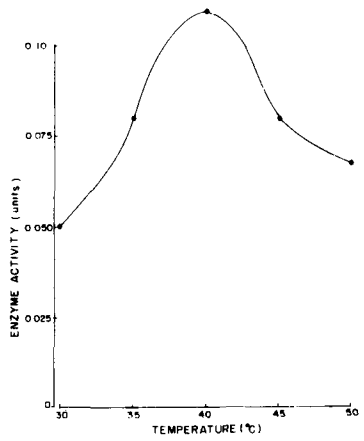

FIG. 5. Determination of optimal temperature of phospholipase A activity in the 20,000 × g pellet from *N. crassa*. The assay mixture consisted of 1 ml egg yolk suspension, 0.5 ml enzyme preparation (20 μg protein) and 4.5 ml 0.9% NaCl. Incubation was carried out for 10 min.

LPC formed by hydrolysis of 1-stearyl-2-oleoyl lecithin (PC) by the 20,000 × g pellet contained 33.8% stearic and 66.1% oleic acid indicating that 66.1% activity resulted from phospholipase A_1 (EC 3.1.1.32) and 33.8% activity resulted from phospholipase A_2 (EC 3.1.1.4).

Lysophospholipase Activity

Enzyme reactions were carried out for 1, 3 and 12 hr as described in assay method B using 20,000 × g pellet (1 mg protein/ml). The aqueous layer was analyzed by paper chro-

TABLE II

Influence of Several Activators and Inhibitors on Phospholipase A Activity

Activators and inhibitors	Concentration	Activation (%)	Inhibition (%)
Calcium ion	16.6×10^{-4} M	31.2	–
Magnesium ion	16.6×10^{-4} M	12.5	–
Cadmium ion	12.5×10^{-4} M	–	100
Zinc ion	8.3×10^{-5} M	–	100
Ethylene diamine tetraacetic acid	16.6×10^{-4} M	–	100
Diethyl ether	1.7% (v/v)	19.0	–
Ethanol	1.7% (v/v)	–	8.5
Triton X-100	0.1% (v/v)	–	59.3
Sodium deoxycholate (DOC)	0.1% (v/v)	17.4	–

Influence of the activators and inhibitors were determined by assay method A except that of Triton X-100 and DOC which were determined by using assay method B.

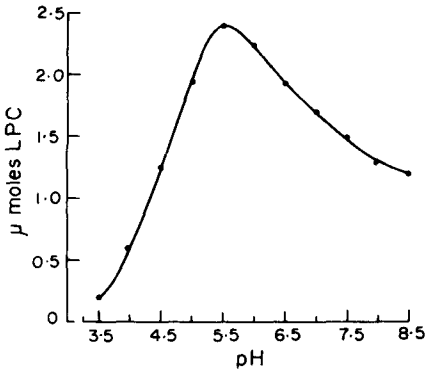

FIG. 6. Determination of optimal pH of phospholipase A activity in the 20,000 × g pellet from *N. crassa*. The assay mixture consisted of 4.08 μmol synthetic PC dissolved in 1 ml diethyl ether/methanol (95:5, v/v), 0.5 ml enzyme preparation (1 mg protein), 0.5 ml 0.2 M buffer (acetic acid-sodium acetate buffer [pH range 3.5-5.5] or citrate-phosphate buffer [pH range 6-7] or boric acid-borax buffer [pH range 7.5-8.5]), and 0.1 ml 1 M calcium chloride solution, and incubated at 40 C for 3 hr.

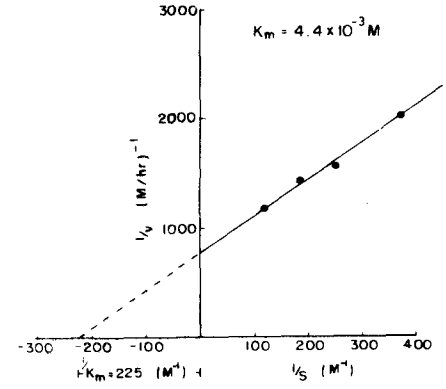

FIG. 7. Effect of substrate concentration (s) on initial rate of hydrolysis (v) of L-α-dipalmitoyl PC (synthetic) by the phospholipase A in the 20,000 × g pellet. The reaction mixture consisted of PC dissolved in 1 ml diethyl ether/methanol (95:5, v/v), 0.5 ml enzyme preparation (1 mg protein), 0.5 ml 0.2 M acetic acid-sodium acetate buffer (pH 5.5), 0.1 ml 1 M calcium chloride solution and incubated at 40 C. Initial rate of PC hydrolysis was determined by estimating the amount of LPC formed during a 1 hr period.

matography for detection of glycerophosphoryl bases. No lysophospholipase activity could be detected up to 3 hr of incubation. However, in the reaction mixture incubated for 12 hr, egg PC in ether and dipalmitoyl PC in ether/methanol (95:5, v/v) were hydrolyzed to GPC. Control experiments showed the absence of nonenzymic formation of GPC. Under similar conditions, egg PE in ether and dipalmitoyl PE in ether/methanol (90:10, v/v) were hydrolyzed to GPE after 12 hr of incubation.

Acyltransferase Activity

Palmitoyl LPC was prepared by the action of 20,000 × g pellet (4.5 mg protein/ml) as described in assay method B. LPC fraction was separated by TLC; 2.01 μmol palmitoyl LPC was sonicated in 0.5 ml of 0.2 M acetic acid-sodium acetate buffer (pH 5.5) in an Ultrasonic Cleaner (Laboratory Supplies Co. Inc.) for 10 min. This was incubated with 0.5 ml of 20,000 × g pellet suspended in 0.9% NaCl (2 mg protein/ml) at 37 C for 4 hr with occasional shaking. The reaction mixture was extracted 3 times with chloroform/methanol (2:1, v/v) and analyzed as described in assay method A. About 48% conversion of LPC to PC was observed. Control experiments showed the absence of endogenous or nonenzymic formation of PC. The remaining aqueous layer after solvent extraction was analyzed by paper chromatography for detection of GPC. No GPC was detected.

Bound Phospholipase A Activity in Mycelia

Six- to 7-day-old defatted mycelia were tested for the presence of phospholipase A activity bound to fungal mycelia by assay method C; 10% hydrolysis of PC to LPC occurred in a 3 hr period. It is evident from control experiments that the net enzymic LPC formation possibly denotes hydrolysis of only substrate PC since the amount of endogenous PC remained unaffected under the conditions of assay.

DISCUSSION

The turbidimetric assay method A has been shown (11) to be specific for phospholipase A activity and has been used in this study to detect this activity in the mycelial homogenate or mycelial subfraction of *N. crassa*. Figure 2 shows that the relationship between enzyme activity and protein concentration is linear up to a certain limit, after which it attains a plateau value. All enzyme activities have been determined with protein concentration in the linear part of the curve. Similar high correlations between enzyme activity in terms of turbidimetric assay units and the products formed as observed here has been reported (11,23). However, this method has some limitation because pH optimum (24), positional specificity and apparent K_m (25) cannot be measured by this method. Therefore, assay

method B, in which phospholipase A activity has been measured by the estimation of net lysophosphatides formed, has been used for these purposes.

Like some other catabolic enzymes (8) of *N. crassa*, the phospholipase A activity also is maximal in late phases of growth. The sp act is considerably higher when the mycelia are disrupted in 0.9% NaCl rather than in 0.25 M sucrose. However, the subcellular particles have a tendency to aggregate as clumps during differential centrifugation in the presence of the 0.9% NaCl but not in the presence of the 0.25 M sucrose (26). For this reason, mycelia has been disrupted in 0.25 M sucrose and the enzymatic properties of the most active fraction have been measured by suspending it in 0.9% NaCl, which is a potential activator (27) of phospholipase A_2-catalyzed reactions.

Table I shows that the total activity is highest in the 110,000 x g supernatant, but the sp act is maximal in the 20,000 x g pellet, indicating the maximal purity of phospholipase A in this fraction. Therefore, all further studies have been carried out with this fraction. Increases in total activity in the soluble supernatant in comparison to the mycelial homogenate may result from removal of some inhibitors during differential centrifugation. Similar observations have been reported previously (28).

The fractions obtained during differential centrifugation are identified as follows: the 12,000 x g pellet fraction is designated a mitochondrial fraction (29,30). It has not yet been possible to obtain any definite idea about the exact nature of the 20,000 x g pellet. This may be identical to the subcellular protease particles isolated from *N. crassa* (28) or may represent fungal lysosome (31). The 110,000 x g pellet may be designated a microsomal fraction by analogy with similar organisms (32).

Increases in phospholipase A activity in the 20,000 x g pellet by sonic disintegration possibly indicates its bound nature like that of *Dictyostelium* (33). EDTA inhibits the phospholipase activity in the 20,000 x g pellet. The addition of Ca^{++} ion does not restore the activity of the enzyme and the formation of a nondissociable and inactive enzyme-Ca^{++}-EDTA complex is suggested (11).

Phospholipase D is active in the presence of the detergents used.

The Michaelis constant (K_m) has little value as a constant measuring enzyme-substrate affinity in biphasic phospholipolytic reactions. However, in such reactions, the applicability of Michaelis-Menten kinetics has often been examined (34-36) and the term "apparent K_m" is generally used (5). Here, also, apparent K_m is ca. 4.4×10^{-3} M.

In studies on the presence of phosphoglyceride acyl transferase activity in the 20,000 x g pellet, no GPC formation could be detected. This possibly indicates that under the conditions described, the formation of PC from LPC was brought about by the acyl transferase reaction, LPC + fatty acyl CoA = PC (37), rather than by the reaction 2 LPC = PC + GPC (38-40). In this situation, endogenous fatty acids and cofactors, e.g., possibly take part in the acyl transferase reaction. Phospholipase A and acyl transferase apparently constitute the Lands Cycle (41) in the 20,000 x g pellet of *N. crassa*.

Like many other insoluble phospholipases (35,42), attempts to solubilize and isolate the *Neurospora* enzymes in a very pure state by using conventional preparatory techniques have not been very successful. However, detergents do have some adverse effects, e.g., change in apparent affinity of the enzyme for the substrate (34), and formation of large aggregates elutable in the void volume of the Sephadex G-200 column (42). Purification of the intracellular phospholipases is complicated possibly because they usually are bound to membranous structures (43).

ACKNOWLEDGMENT

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Selectivity in Incorporation, Utilization and Retention of Oleic and Linoleic Acids by Human Skin Fibroblasts[1]

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ABSTRACT

Fetal human fibroblasts were grown in culture medium containing 10% fetal bovine serum supplemented with [1-^{14}C] linoleate or [1-^{14}C] oleate. At all concentrations of exogenous fatty acids, the incorporation of oleate was greater than that of linoleate. With increased medium fatty acid concentrations, linoleate in triacylglycerol (TAG) could be increased from 13 to 75% of the total incorporated; at each concentration, relatively more linoleate than oleate was in TAG. When the cells were exposed to exogenous oleate/linoleate mixtures, the composition of the mixture determined the extent of incorporation of both fatty acids. When the mixture was primarily linoleate, scarce oleate was used preferentially for phospholipids (PL); no such specificity for scarce linoleate was observed. Addition of exogenous fatty acids resulted in a shift of previously incorporated ^{14}C fatty acids from phospholipid into TAG; retention of oleate in PL was greater than that of linoleate. Incorporation of oleate into phospholipids was also higher than that of linoleate from exogenous fatty acid mixtures which were 80% saturated. It is suggested that normal human fibroblasts have adapted to the low levels of exogenous polyunsaturated fatty acids in culture media by increased use of oleate in phospholipid. Even when the cells are supplemented with linoleate, the preferential use of oleate in phospholipid groups is retained.

INTRODUCTION

Mammalian cells in culture normally obtain much of their fatty acids from serum in the growth medium (1,2), and their fatty acid composition reflects that of the medium (3). Fetal bovine serum, used in culture media for many serum-requiring cell lines, is very low in essential fatty acids (4). Cells in culture exhibit lower polyunsaturated fatty acid (PUFA) levels, principally linoleate plus arachidonate, and higher levels of oleate than do corresponding tissues in vivo (5). Growth of cells in medium without lipids results in a further decrease in PUFA and an increase in oleate (6).

Human skin fibroblasts readily incorporate exogenous fatty acids into cellular lipids (7,8), and exchange previously incorporated fatty acids with those in the culture medium (7). Like transformed cell lines, their fatty acid composition can be modified extensively by medium supplementation with fatty acids (9). Furthermore, these cells respond to increased medium concentrations of free fatty acids by synthesizing triacylglycerol droplets (10), thus providing an alternative metabolic fate for incorporated fatty acids.

This study examines the specificity of linoleate and oleate use by GM-10 fibroblasts grown in medium containing fetal bovine serum. It was hypothesized that these cells might exhibit preferential incorporation of linoleic acid, and that, especially when linoleic acid was relatively scarce, it might be incorporated into phospholipid rather than triacylglycerol. Our results indicate, however, that under a wide variety of experimental conditions, there is a small, but consistently significant preference for oleate rather than linoleate in phospholipid synthesis.

MATERIALS AND METHODS

Cell Culture and Fatty Acid Supplementation

GM-10 cells, human diploid fibroblasts derived from skin of a 3-month fetus (General Mutant Cell Repository, Camden, NJ), were propagated in Eagle's Minimum Essential Medium, supplemented with 10% noninactivated fetal bovine serum (both from Grand Island Biological Co., Grand Island, NY) at 37 C in a humidified, 5% CO_2 atmosphere. Replicate 25-cm^2 flasks were seeded with 3-4 × 10^5 cells in 4.0 ml medium; all experiments used cells between 15 and 30 generations in culture. Medium supplementation with fatty acids was done 24 hr after subculture, when the cells were actively mitotic (7).

[1-^{14}C] Linoleic acid (51.0 mCi/mmol) and [1-^{14}C] oleic acid (48.0 mCi/mmol) were obtained from New England Nuclear Corp., Boston, MA. Radiolabeled fatty acids were greater than 99% free fatty acid as determined by thin layer chromatography (TLC). Upon methylation with Boron trichloride/methanol, gas liquid chromatography (GLC) and ^{14}C detection using a Packard 804 Gas Flow Proportional Counter, the [^{14}C] oleate and [^{14}C]-

[1]Presented in part at the ASBC Meeting, Dallas, April 1979.

linoleate were 94-97% pure; no labeled impurity represented more than 1.0% of the total radioactivity. Linoleic acid (Grade III) and oleic acid (Sigma grade) from Sigma (St. Louis, MO) were greater than 98% pure by GLC. Fatty acid solutions were stored in hexane under N_2 at -20 C; concentrations were confirmed by titration (11). For each experiment, aliquots of fatty acid and [^{14}C] fatty acid solutions were evaporated to dryness under N_2 and redissolved in dilute NaOH. The resulting sodium salts were filter sterilized and added to serum before dilution with culture medium. Alternatively, solutions of fatty acids in 95% ethanol were added directly to the serum; the final ethanol concentration in the culture medium was 0.2%.

Delipidized fetal bovine serum, prepared by acetone/ethanol (1:1, v/v) extraction according to Rothblat et al. (12), was used to replace fetal bovine serum in some experiments; extraction was 93% effective as determined by GLC. Protein standardization of the solvent extracted serum was done according to Lowry et al. (13). When 1.3 μg/ml α-tocopherol (phosphoric acid ester, disodium salt, ICN, Cleveland, OH) was added to the cultures, it was solubilized in pluronic F-68 (BASF Wyandotte Corp., Wyandotte, MI), with a final detergent concentration of 0.025% (14).

Lipid Extraction

After growth with supplemented fatty acids, usually for 24 hr, the cells were washed twice with calcium- and magnesium-free Earle's Basic Salt Solution and detached from the surface with 1.5 ml/flask of 0.05% trypsin in Earle's Salts (GIBCO). Each cell suspension in trypsin was transferred to a 40-ml conical tube containing 4.0 ml acetone plus 2 μg α-tocopherol; 2.5 ml of 0.025% methyl cellulose in 0.9% NaCl was used to rinse each flask. Cellular lipids were then extracted directly in a mixture of ethyl acetate/acetone/cell suspension (2:1:1 v/v) (15). Medium samples were extracted in 6 vol of ethyl acetate/acetone (2:1). Each sample was mixed, heated to 85 C, vortexed vigorously and incubated at 65 C for 40 min with a 1 min vortexing at 20 min. The solvent mixture was then centrifuged at 2000 rpm at 4 C for 10 min, and the organic phase removed and stored at -20 C under N_2 for subsequent analysis.

Thin Layer Chromatography and Scintillation Counting

Aliquots of the lipid extracts were redissolved in small volumes of chloroform/methanol (1:1, v/v) and applied to Silica Gel H coated thin layer chromatographic plates (Applied Science, State College, PA). The plates were developed by ascending chromatography in petroleum ether/diethyl ether/acetic acid (82:18:1, v/v) for separation of neutral lipid classes. After removal of phospholipids at the origin, the neutral lipid spots were visualized with iodine. Duplicate plates were developed in chloroform/methanol/acetic acid/water (50:30:8:4, v/v) for separation of phospholipids. Appropriate reference mixtures were cochromatographed with the samples.

Aqueous samples were assayed for radioactivity in 1.0 ml ACS (Amersham, Arlington Heights, IL). Samples of lipid extracts and neutral lipid spots were dissolved in 15 ml of 0.4% Omnifluor (New England Nuclear) in toluene/ethanol (2:1, v/v). Phospholipid spots were redissolved in 1.0 ml water to which ACS scintillation fluid was then added; radioactivity measurements were done with a Beckman LS-250 liquid scintillation counter.

RESULTS

Incorporation of [1-^{14}C] Linoleate and [1-^{14}C] Oleate

GM-10 fibroblasts in monolayer culture readily take up both [1-^{14}C] linoleate and [1-^{14}C] oleate from medium containing fetal bovine serum. Table I shows that, in a representative experiment, 62.5% of the [1-^{14}C]-oleate and 50.8% of the [1-^{14}C] linoleate were incorporated in 24 hr. In this system, incorporation of exogenous fatty acids is linear with time for at least 6 hr and continues to increase for over 48 hr; the apparently diminished incorporation rate after 6 hr results from turnover and release of incorporated acyl groups (10). Incorporation varied somewhat with cell density, cell generations in culture and the lot of fetal bovine serum. Uptake of 0.5-1.0 μCi/flask [1-^{14}C] linoleate was lower than that of [1-^{14}C] oleate in each of 13 independent experiments with 3 different lots of fetal bovine serum, with a significance of $p < 0.001$ (paired t-test).

The lipid extracts contained 95% of the incorporated [1-^{14}C] linoleate or [1-^{14}C] oleate; 96% of the [^{14}C] lipids were phospholipids and triacylglycerols. In the experiment shown in Table I, 21% of the [^{14}C] lipid was triacylglycerol for linoleate-fed cells, but only 14% for oleate-fed cells; the localization of relatively more of the incorporated [1-^{14}C] linoleate in triacylglycerol was significant at $p < 0.001$ for the series of 13 experiments. Neither addition to the medium of the fatty acids in ethanol rather than as aqueous soaps, nor the presence

TABLE I

Incorporation of Low Levels of [1-^{14}C] Linoleate and [1-^{14}C] Oleate by GM-10 Fetal Human Fibroblasts[a]

Fatty acid added	[1-^{14}C] Linoleate			[1-^{14}C] Oleate		
	10^{-3} X dpm/flask	%	nmol/flask[c]	10^{-3} X dpm/flask	%	nmol/flask[c]
Total incorporation	1,042.3 ± 19.1	50.8	13.5	1,283.1 ± 25.4	62.5[b]	27.3
Distribution of ^{14}C in cells						
Lipids	985.8 ± 11.2	94.6	12.8	1,221.1 ± 4.3	95.2	26.0
Aqueous phase and pellet	56.0 ± 1.3	5.4	0.7	61.9 ± 1.6	4.8	1.3
Distribution of ^{14}C in lipids						
Phospholipid	738.7 ± 8.6	74.9	9.6	1,011.2 ± 11.9	82.8[b]	21.5
Diacylglycerol	13.4 ± 0.3	1.4	0.18	12.4 ± 0.5	1.0	0.26
Free fatty acid	8.6 ± 0.1	0.9	0.12	11.7 ± 0.3	1.0	0.26
Triacylglycerol	208.1 ± 3.5	21.1	2.70	172.2 ± 3.2	14.1[b]	3.67
Cholesterol esters	17.8 ± 0.2	1.8	0.23	13.6 ± 0.3	1.1[b]	0.29
Distribution of ^{14}C in phospholipids						
Lysophosphatidylcholine	4.1 ± 0.1	0.6		5.0 ± 0.2	0.5	
Sphingomyelin	16.3 ± 0.7	2.2		19.9 ± 0.6	2.0	
Phosphatidylcholine (PC)	520.8 ± 4.7	70.5		743.4 ± 2.2	73.5[b]	
Phosphatidylserine plus phosphatidylinositol	80.6 ± 1.9	11.0		99.1 ± 0.4	9.8[b]	
Phosphatidylethanolamine (PE)	52.0 ± 0.6	7.0		91.9 ± 1.2	9.1[b]	
Acidic phospholipids	65.2 ± 1.1	8.8		52.4 ± 0.3	5.2[b]	
PC/PE	10.0 ± 0.2			8.1 ± 0.2[b]		

[a]GM-10 fibroblasts were seeded at 3 X 10^5 cells/25-cm^2 flask. After 24 hr, the culture medium was replaced with 4.0 ml Eagle's Minimal Essential Medium supplemented with 10% fetal bovine serum plus either 1.0 μCi [1-14C]oleate (48.0 mCi/mmol) or 1.06 μCi [1-^{14}C]linoleate (51.0 mCi/mmol). Incubation time with radiolabeled fatty acids was 24 hr. Data are means ± SE of determinations on 3 replicate flasks.

[b]Difference between values for [1-^{14}C]oleate and [1-^{14}C]linoleate was significant at $p < 0.05$ in this representative experiment. In paired comparisons of 13 independent experiments, the differences between the 2 [^{14}C]fatty acids were significant at $p < 0.001$ for total incorporation and neutral lipid distribution. Differences in [^{14}C]fatty acid distribution between phospholipid subclasses were significant at $p < 0.01$ in paired comparisons of 4 experiments.

[c]Calculation of total uptake of free fatty acid based on 22.8 nmol oleate and 5.7 nmol linoleate/4 ml medium contributed by the fetal bovine serum. The [^{14}C]-fatty acids were added at 20.8 nmol/flask; total concentrations of [1-^{14}C]linoleate and [1-^{14}C]oleate would thus be 26.5 and 43.6 nmol/flask, respectively.

of α-tocopherol in the medium, significantly altered the results just described. The net effect of both the lower incorporation rate of [1-^{14}C]-linoleate than [1-^{14}C] oleate and the higher percentage of incorporated linoleate in triacylglycerol was that the absolute amount of supplemented linoleate incorporated into phospholipids was less than 75% that of supplemented oleate.

Fetal bovine serum contains 210-280 nmol/ml free fatty acid. The lot used for most of these experiments contributed 22.8 nmol/4 ml oleate and 5.7 nmol/4 ml linoleate when present at 10% in the culture medium. The 20.8 nmol of [1-^{14}C] oleate was thus diluted by the corresponding serum free fatty acid to a greater extent than was the 20.8 nmol [1-^{14}C]-linoleate. On a total mass basis, the greater uptake of oleate than linoleate and the more extensive use of oleate in phospholipids were even more pronounced than indicated by percentage incorporation.

Minimal Fatty Acid Supplementation

To determine if the addition of fatty acids to the medium was responsible for the observed synthesis of triacylglycerol, [1-^{14}C] oleate and [1-^{14}C] linoleate were each added to a series of flasks in decreasing amounts, so that they contributed from 10.0 to 0.9 nmol/ml fatty acid to the culture medium. The results (Fig. 1) indicated that the differences between oleate and linoleate incorporation remained with decreased concentrations of medium supplementation, and, by extrapolation, should occur with a zero level of fatty acid supplementation. Thus, at this density, GM-10 cells would incorporate 57% of the serum oleate (13.0 nmol/flask) and 50% of the serum linoleate (2.85 nmol/flask) in 24 hr.

Figure 1B shows that with decreased exogenous [^{14}C] fatty acid concentrations, the extent of use of incorporated fatty acids in triacylglycerol decreased. The percentage of incorporated [1-^{14}C] linoleate in triacylglycerol remained higher than that of [1-^{14}C] oleate. By extrapolation, 13% of the linoleate and 9% of the oleate incorporated from medium with 10% fetal bovine serum alone would be in triacylglycerol. On a mass basis, however, oleate incorporation into triacylglycerol (0.78 nmol/flask) would be greater than that of linoleate (0.37 nmol/flask). Apparently, synthesis of triacylglycerol from exogenous fatty acids is a normal occurrence for GM-10 fibroblasts grown in medium with fetal bovine serum. Furthermore, both the relatively greater incorporation of oleate than linoleate, and the use of

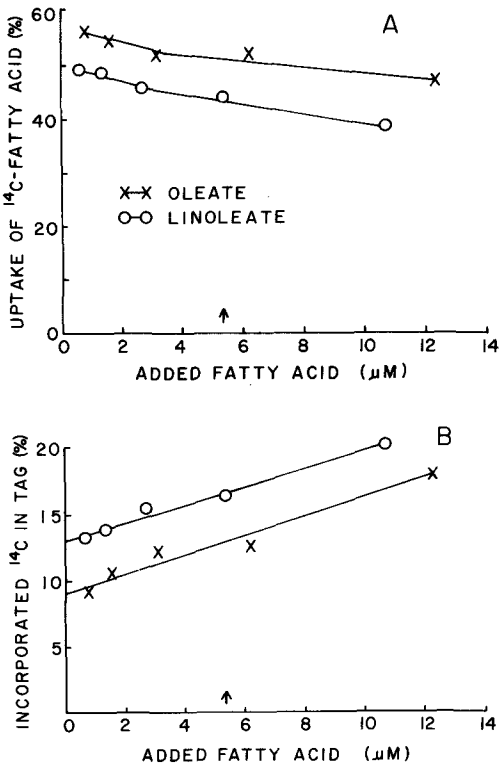

FIG. 1. Effect of minimal medium supplementation with fatty acids on incorporation of [1-^{14}C]-linoleate and [1-^{14}C] oleate. GM-10 fibroblasts were seeded at 2.5 X 10^5 cells/flask. Replicate flasks were incubated for 24 hr with 0.14-2.5 μCi [1-^{14}C] linoleate or [1-^{14}C] oleate as described in Table I. A: Uptake of [^{14}C] fatty acid in triacylglycerol (TAG). **O–O**, [1-^{14}C] linoleate; **X–X** [1-^{14}C] oleate. The arrows indicate the added [^{14}C] fatty acid concentration used in Table I.

a higher percentage of incorporated linoleate in triacylglycerol, suggest that despite the low concentration of linoleate in fetal bovine serum, there is a lack of preferential use of serum linoleate in phospholipids.

We also examined incorporation of 2.7 μM fatty acid from 4.0 ml of culture medium in which the fetal bovine serum was replaced with delipidized serum protein. Incorporation of [^{14}C] oleate was 9.19 nmol/flask (mean of 3 independent experiments); that of linoleate was 9.05 nmol/flask. Of the incorporated fatty acids, 8.72 nmol oleate and 8.40 nmol linoleate were in phospholipid, whereas 0.25 nmol oleate and 0.29 nmol linoleate were in triacylglycerol. Even with fatty acid concentrations below those of serum-supplemented media, there was greater use of oleate than linoleate in cellular phospholipids.

Effect of Saturated Fatty Acids on Incorporation of Oleate and Linoleate

Phospholipids in vivo commonly contain one saturated and one polyunsaturated fatty acid. To determine if the incorporation of linoleate into phospholipids by human fibroblasts would be enhanced by saturated fatty acids, replicate flasks were incubated with mixtures of oleate and linoleate plus either palmitate or stearate in medium containing delipidized fetal bovine serum; each fatty acid was ^{14}C-labeled in turn. As shown in Table II, the exogenous fatty acids were not incorporated into cellular lipids in quite the same proportions as supplied in the medium. For example, with increased saturation of the exogenous mixture, the percentage of oleate uptake increased and that of palmitate decreased. In all cases, however, the extent of linoleate incorporation was less than that of oleate. With 50% saturated fatty acids, the percentage use of linoleate was less than oleate at a ratio of exogenous oleate-to-linoleate of either 1:4 or 4:1. The more extensive uptake of oleate than linoleate was reflected directly in a greater incorporation, on a mass basis, of oleate into phospholipid when the two were equimolar in the medium. Thus, even when there is extensive use of saturated fatty acids, oleate is the preferred unsaturated fatty acid for phospholipid synthesis in these cells.

Saturated fatty acids promote less triacylglycerol synthesis than do unsaturated ones (M.D. Rosenthal, unpublished data). Although there was little accumulation of neutral lipids, small amounts of all [^{14}C]fatty acids were located in triacylglycerol. Incorporation of fatty acids on a mass basis indicated more oleate than linoleate in triacylglycerol from equimolar exogenous mixtures (data not shown). Expressed as a percentage of incorporated fatty acid in TAG, however, the two were similar at ca. 4% in 80% palmitate and 10% in 80% stearate mixtures.

Increased Medium Fatty Acid Concentrations

Higher exogenous free fatty acids concentrations resulted in increased incorporation of both oleate and linoleate; incorporation on a percentage basis decreased less than 2-fold with the 80-fold increase in concentration (Table III). Oleate incorporation was higher than that of linoleate, both in terms of the supplemented fatty acid alone (percentage basis) and total nmol/flask, including the contribution of serum free fatty acids. With increased oleate or linoleate accumulation, discrete triacylglycerol droplets became appar-

TABLE II

Incorporation from Mixtures of Saturated and Unsaturated Fatty Acids

	Exogenous fatty acids (nmol/flask)			Incorporation into lipid[b] (nmol/flask [%])			Incorporation into phospholipid		
	16:0	18:1	18:2	16:0	18:1	18:2	16:0	18:1	18:2
Expt. I									
	70.0	8.8	8.8	27.2 (38.8)	3.65 (41.5)	2.81 (31.9)	22.2	3.44	2.60
	43.8	35.2	8.8	20.1 (46.0)	12.0 (34.1)	2.44 (27.7)	17.9	10.4	2.12
	43.8	8.8	35.2	20.5 (46.8)	3.19 (36.3)	8.62 (24.5)	18.2	2.83	7.18
	18:0	18:1	18:2	18:0	18:1	18:2	18:0	18:1	18:2
Expt. II									
	70.0	8.8	8.8	24.2 (34.6)	3.77 (42.8)	2.93 (33.3)	19.0	3.24	2.54

[a]Log phase GM-10 cells were washed 2X with EBSS and incubated for 24 hr with 5.0 ml MEM with 5% delipidized serum protein in EBSS, plus 17.6 μM fatty acid mixture, composition as indicated. Each fatty acid was radiolabeled in one set of flasks. Abbreviations: 16:0, palmitate; 18:0, stearate; 18:1 oleate; 18:2, linoleate.

[b]Incorporation of each [^{14}C] fatty acid expressed as nmol/flask, and as a percentage of that supplied in the medium. Values are means, n = 3.

TABLE III

The Effect of Increased Medium Free Fatty Acid Concentrations on Incorporation of [1-^{14}C]Oleate and [1-^{14}C]Linoleate[a]

Fatty acid	Concentration		Total incorporation		Triacylglycerol		Phospholipid	
	Supplemented (nmol/flask)	Total[b] (nmol/flask)	(nmol/flask [%])		(% of incorp.)	(nmol/flask)	(% of incorp.)	(nmol/flask)
[1-^{14}C]Oleate	9.7	32.5	56.8	18.5	15.2	2.8	80.4	14.9
	44.4	67.2	53.1	36	21.8	7.8	74.2	26.7
	79.2	102	52.0	53	28.4	15	65.3	35
	149	172	47.9	82	40.0	33	55.1	45
	288	311	41.2	128	52.3	67	43.0	55
	565	588	38.7	228	62.6	143	31.9	73
	843	866	34.0	294	72.7	214	23.6	69
	1121	1144	37.2	425	79.8	340	16.6	71
	1399	1422	32.7	465	80.8	376	15.1	70
[1-^{14}C]Linoleate	9.7	15.4	46.1	7.1	18.7	1.3	76.2	5.4
	44.4	50.1	40.8	20.4	30.7	6.3	61.7	12.6
	79.2	84.9	40.3	34	40.8	14	52.5	18
	149	155	30.1	47	49.8	23	41.5	20
	288	294	24.8	73	60.0	44	33.0	24
	565	571	25.2	144	74.8	108	18.0	26
	843[c]	851	–	–	–	–	–	–

[a]Replicate flasks of log phase GM-10 cells were seeded with 2.5 X 10^6 cells. After 24 hr, the culture medium was replaced with 4.0 ml MEM plus 10% FBS supplemented with 0.5 μCi/flask [1-^{14}C]oleate or [1-^{14}C]linoleate, plus increased concentrations of the respective nonlabeled fatty acid. Values are means from 2 experiments.

[b]Total medium free fatty acid concentrations including the 22.8 nmol/flask oleate and 5.7 nmol/flask linoleate provided by the serum.

[c]Linoleate concentrations above 200 μM were toxic in this series.

ent in the cytoplasm; similar lipid droplets have been described in L cells (6). The GM-10 cells retained their characteristic morphology except at the highest medium fatty acid concentrations. The accumulation of lipid droplets was accompanied by esterification of a greater percentage of the incorporated fatty acids into triacylglycerol; at comparable exogenous fatty acid concentrations, relatively more of the incorporated linoleate than oleate was esterified into triacylglycerol. Thus, at these higher fatty acid concentrations as well, GM-10 cells use oleate in phospholipids to a far greater extent than linoleate.

Use of Oleate/Linoleate Mixtures

To extend the observation that linoleate and oleate were used differently from free fatty acid mixtures such as that of fetal bovine serum, GM-10 cells were incubated with mixtures of oleate and linoleate, with a total supplemented fatty acid concentration of 140 μM, resulting in substantial triacylglycerol accumulation while maintaining viable, actively mitotic cells. As in Table III, when a single fatty acid was added, incorporation of [1-^{14}C]-oleate was greater than that of [1-^{14}C] linoleate. From any given fatty acid mixture, however, the percentage incorporation of oleate and linoleate were identical (Fig. 2A). Thus, the incorporation of [1-^{14}C] oleate was depressed when it was diluted with linoleate rather than oleate.

Figure 2B shows that in oleate/linoleate mixtures which were 95-97.5% oleate, distribution of incorporated [1-^{14}C] oleate and [1-^{14}C] linoleate between phospholipid and triacylglycerol was similar. With increased linoleate in the fatty acid mixture, the percentage of incorporated [1-^{14}C] linoleate in phospholipid declined whereas that of [1-^{14}C]-oleate did not. Thus, when exposed to excess linoleate, these cells preferentially used scarce oleate for phospholipid and stored relatively more of the incorporated linoleate in triacylglycerol. Similar results (not shown) were obtained when the oleate/linoleate mixtures were added to the culture medium with delipidized serum protein rather than fetal bovine serum. The ratio of [1-^{14}C] oleate in phosphatidylcholine to that in phosphatidylethanolamine (PC/PE) decreased from 6.4 in 100% oleate to 5.0 when the [1-^{14}C] oleate was diluted with linoleate. The PC/PE ratio of the incorporated [1-^{14}C] linoleate remained 7.1-7.2 throughout the range of mixtures. These data suggest that when oleate is relatively scarce, it is used preferentially for PE and also for phosphatidylinositol plus phosphatidylserine.

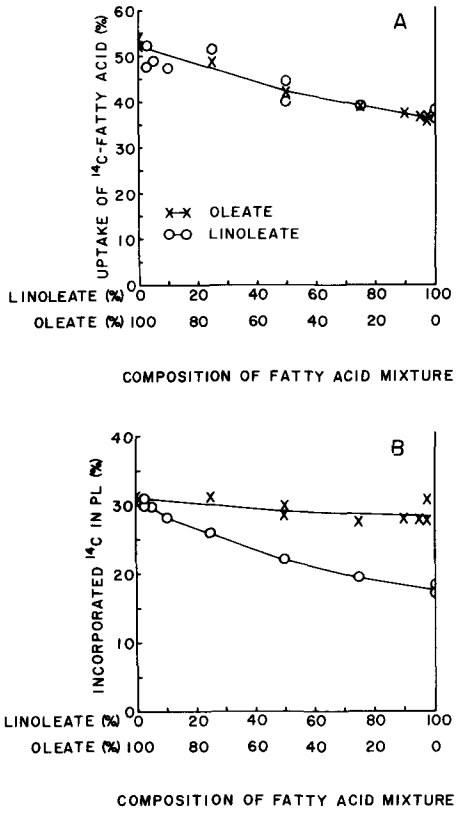

FIG. 2. Effect of different oleate/linoleate mixtures on incorporation of [1-^{14}C] linoleate and [1-^{14}C]-oleate. Replicate flasks were incubated for 24 hr in MEM plus 10% FBS plus 0.5 μCi [1-^{14}C] linoleate or [1-^{14}C] oleate, plus nonradiolabeled fatty acids to result in the indicated oleate/linoleate mixtures. Total supplemented fatty acid concentration was 140 μM. Uptake of [^{14}C] fatty acid as a percentage of that added to the medium. B: Percentage of incorporated [^{14}C] fatty acids in phospholipids (PL). O--O, [1-^{14}C]-linoleate; X--X, [1-^{14}C] oleate.

Pulse-chase Studies

In order to examine the metabolism of linoleate and oleate after incorporation into cellular lipids, cells were labeled with the respective [^{14}C] fatty acids and then grown in medium without radioactivity. Table IV shows that medium supplementation with 100 μM fatty acid greatly enhanced release of previously incorporated [^{14}C] fatty acids. Nearly all of this increase was in the form of free fatty acids; more [^{14}C] fatty acids were released with linoleate supplementation than with oleate. In the absence of fatty acid supplementation, [1-^{14}C] oleate and [1-^{14}C] linoleate were released to a similar extent. With addition of 100 μM fatty acid, release of [1-^{14}C] oleate was greater than that of [1-^{14}C] linoleate. Although

TABLE IV

Effect of Added Oleate and Linoleate on Release of Previously Incorporated [1-^{14}C] Fatty Acid

Released fatty acid (10^{-3} × dpm/flask	Total[b]	Phospholipid[e]	Free fatty acid[e]	Aqueous phase[e]
[1-^{14}C] Linoleate:				
Control	76.4 ± 0.3	13.7	17.1	42.5
Added oleate	170.3 ± 2.3	13.1	110.2	38.3
Added linoleate	228.3 ± 1.1[d]	14.4	162.1	43.1
[1-^{14}C] Oleate:				
Control	64.4 ± 1.4	11.0	17.0	33.8
Added oleate	205.6 ± 1.7[c]	14.4	156.8	29.6
Added linoleate	264.5 ± 3.5[c,d]	15.4	208.9	33.9

[a]Replicate flasks of GM-10 cells were incubated for 24 hr with 0.65 μCi/flask [1-^{14}C] linoleate or [1-^{14}C]-oleate as described in Table I; initial corporation was 778.2 × 10^3 and 798.3 × 10^3 dpm/flask for [1-^{14}C]-linoleate and [1-^{14}C] oleate, respectively. Each flask was then rinsed twice with sterile EBSS and the cells incubated for a second 24 hr in MEM plus 10% FBS (control) with 100 μM fatty acid added as indicated.

[b]Total medium radioactivity; means ± SE, n = 3.

[c]Difference between values for [1-^{14}C] linoleate and [1-^{14}C] oleate, with released fatty acid expressed as a percentage of initial incorporation, has $p < 0.05$.

[d]Difference between effects of added oleate and added linoleate has $p < 0.05$.

[e]2.0 ml of medium from each flask was extracted with 4.0 ml acetone and 8.0 ml ethylacetate as described in Materials and Methods. The aqueous phase was separated and the lipids analyzed by TLC.

absolute levels of [^{14}C] fatty acid release varied with cell density and age of the culture, the differences observed in Table IV, and Table V were significant in each of 3 independent experiments.

Medium supplementation with fatty acids resulted in transfer of previously incorporated [1-^{14}C] linoleate and [1-^{14}C] oleate from phospholipid to triacylglycerol (Table V). With either fatty acid in the chase medium, relatively more [1-^{14}C] linoleate was transferred than was [1-^{14}C] oleate. Although more [1-^{14}C]-oleate was released as free fatty acid, retention of [1-^{14}C] oleate in phospholipids was greater than that of [1-^{14}C] linoleate. This difference was quite pronounced with the linoleate chase; 44.0% of the initially incorporated [1-^{14}C] oleate but only 35.1% of the [1-^{14}C] linoleate remained in phospholipid. The losses of both [^{14}C] fatty acids from phospholipids occurred disproportionately from PC. These results, like those obtained with use of oleate/linoleate mixtures, indicate the preferential use of oleate for phospholipids when diploid human fibroblasts are challenged with excess exogenous linoleic acid.

DISCUSSION

These studies have demonstrated that GM-10 normal human skin fibroblasts take up and esterify oleate somewhat more readily than linoleate from medium containing either fetal bovine serum or delipidized serum protein. Of the incorporated fatty acids, relatively more linoleate is esterified in triacylglycerol; especially when it is limited, oleate is used preferentially for phospholipid. By contrast, Yoshida sarcoma cells preferentially incorporate linoleate rather than oleate from a variety of exogenous fatty acid mixtures (16). The rate of esterification of linoleate into phospholipids by Ehrlich ascites tumor cells is greater than that of oleate (17). Although L 1210 murine leukemia cells incorporate oleate more extensively than linoleate (18), the excess oleate is in triacylglycerol, with similar amounts of the 2 fatty acids incorporated into phospholipid. Since the studies on tumor cells used fatty-acid-poor albumin in place of serum, our results may result from differences in growth conditions and/or those between normal and transformed cells.

Both exogenous fatty acids (6) and hypertriglyceridemic serum (19) stimulate triacylglycerol accumulation in culture. Spector et al. (9) have demonstrated that exogenous fatty acids stimulate triacylglycerol synthesis whereas cellular phospholipid and cholesterol content is unchanged. Supplementation of medium containing fetal bovine serum with up to 100 nmol/ml oleate or linoleate does not affect the growth rate of these cells. Although exogenous fatty acids modify the acyl profile of cellular triacylglycerol to a greater extent than that of phospholipid (9), this study indicates that at any concentration of exogenous fatty acid, relatively more of the incorporated linoleate than oleate is esterified into triacylglycerol. Furthermore, the data suggest that, of those

TABLE V

Effect of Added Oleate and Linoleate on Redistribution of Previously Incorporated [^{14}C] Fatty Acids[a]

Lipid class	[1-^{14}C] Linoleate				[1-^{14}C] Oleate			
	Initial incorporation	After 24 hr chase in:			Initial incorporation	After 24 hr chase in:		
		Control	Oleate	Linoleate		Control	Oleate	Linoleate
Neutral lipid (NL)	112.4 ± 0.2	52.5 ± 0.7	280.3 ± 2.8	266.2 ± 0.8	83.8 ± 1.0	34.5 ± 1.4	238.0 ± 0.8	182.6 ± 3.3
Phospholipid (PL)	665.8 ± 0.9	649.3 ± 1.8	327.1 ± 3.9	273.2 ± 0.3	714.5 ± 8.8	699.4 ± 2.8	354.7 ± 2.5	351.1 ± 0.2
PL (% of PL + NL)	85.6	92.5	53.9	50.6[c]	89.5[b]	95.3[b]	59.8[b]	65.8[b,c]
PL (% of initial PL + NL)	85.6	83.4	42.0	35.1[c]	89.5[b]	87.6[b]	44.4	44.0[b]
Phosphatidylcholine (PC)	520.0 ± 4.1	458.1 ± 9.6	217.6 ± 11.1	171.1 ± 0.2	557.9 ± 1.9	503.9 ± 5.2	265.5 ± 3.7	223.0 ± 2.2
Phosphatidylserine plus phosphatidylinositol	76.9 ± 1.6	92.3 ± 3.7	49.2 ± 1.1	37.0 ± 0.4	69.7 ± 2.8	79.0 ± 0.3	39.7 ± 1.0	48.8 ± 3.3
Phosphatidylethanolamine (PE)	50.6 ± 0.3	77.6 ± 7.0	49.4 ± 1.9	45.8 ± 40.1	68.9 ± 0.5	92.0 ± 1.4	49.3 ± 3.2	60.0 ± 1.0
PC/PE	10.3	5.9	4.4	3.7[c]	8.1[b]	5.5	5.2[b]	3.7[c]

[a]Experimental conditions as in Table IV.

[b]Difference between values for [1-^{14}C] linoleate and [1-^{14}C] oleate has $p < 0.05$.

[c]Difference between effects of added oleate and added linoleate has $p < 0.05$.

free fatty acids normally present in fetal bovine serum, a higher percentage of the linoleate incorporated into cellular lipids should be in triacylglycerols.

Stern and Pullman (5) have shown that adaptation of hepatocytes to culture involves loss of mitochondrial acyl-CoA: glycerol-3-phosphate acyl transferase. Reliance on the microsomal enzyme enables those cells to synthesize dimonounsaturated phospholipids and thus maintain membrane fluidity despite a scarcity of polyunsaturated fatty acids. The hepatocytes continue to esterify oleate instead of saturated fatty acids in the 1-position of phosphoglycerides even when the culture medium is supplemented with linoleate. A similar enzymatic adaptation in human skin fibroblasts might explain the preferential use of scarce oleate in phospholipid. The greater incorporation of oleate than linoleate from mixtures of 80% palmitate or stearate would suggest that these cells also synthesize 1-saturated, 2-monounsaturated phospholipids in preference to the 1-saturated, 2-polyunsaturated species common in vivo.

This study indicates that the preferential use of oleate relative to linoleate in phospholipids is more pronounced in PE than in PC. A similar exclusion of excess polyenoic fatty acids from PE has been reported in L cells (20). Supplementation of L cells with linoleic acid perturbs the unsaturation of phospholipids of the cell homogenate more than those of the plasma membrane; this suggests a cellular mechanism for minimizing possible increases in cell membrane fluidity.

The localization of relatively more incorporated linoleate than oleate in triacylglycerol by GM-10 cells may represent a similar homeostatic mechanism.

A number of relatively normal cell lines apparently require polyunsaturated fatty acids (21-23); studies in which serum is completely replaced with hormones use media which contain linoleic acid (24). No requirement for linoleate has, however, been demonstrated in WI-38 human lung fibroblasts, even at serum protein concentrations of 100 μg/ml (25). Diploid human fibroblasts retain the ability to elongate and further desaturate both linoleate and linolenate (26,27) and to convert arachidonic acid to prostaglandins (28). About 10-15% of incorporated linoleate is modified in the first 24 hr (26); the longer chain polyunsaturated fatty acids are found in both phospholipid and triacylglycerol (Rosenthal, unpublished data). While substantial levels of polyunsaturated fatty acids apparently are not required for growth of these cells in culture, trace amounts of linoleate may be required for prostaglandin synthesis and possibly optimal cell growth. Further investigation is required to determine if cells depleted in polyunsaturated fatty acids might exhibit preferential use of linoleate.

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Microbiological Studies Investigating Mutagenicity of Deep Frying Fat Fractions and Some of Their Components

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ABSTRACT

In this study, the Salmonella/microsome mutagenicity test according to Ames et al. (Mutation Res. 31:347, 1975) was performed in order to detect possible mutagenicity of oxidized deep frying fat fractions. Furthermore, the mono-, di-, tri- and tetrahydroxyoctadecanoic acids and the hydroperoxide of linoleic acid were investigated as model test substances. The Ames assay was carried out with and without metabolic activation including preincubation and liquid culture procedures as described by Mitchell (Mutation Res. 54:1, 1978). The results show no mutagenic effects for the oxidized fractions of deep frying fats nor for the model test substances. At higher concentrations, however, limited test reliability resulted from direct toxic effects on bacterial growth.

INTRODUCTION

Long-term feeding experiments in animals have shown that processed deep frying fats may have deleterious biological effects. Of special toxicological interest are certain fat fractions which arise in considerable amounts during the deep frying process and which can be isolated by analytical methods. These fractions are (a) the polar fraction, according to Guhr and Waibel (1), consisting of dimeric and polymeric triglycerides, and (b) the oxidized fatty acid fraction (2), which is isolated as the petroleum ether insoluble residue. The amount of these fractions serves as the legal basis for determining the acceptability of a given fat for use in Germany. The transesterification of these fat fractions yields a complex mixture in which hydroxy fatty acids can be detected by gas chromatography-mass spectrometry (GC-MS) (3,4). Although this complex mixture has not yet been analyzed completely, mono- and dihydroxy fatty acids were found in considerable amounts (5).

Complementing the toxicological studies in animals, we performed the Salmonella/microsome mutagenicity test according to Ames et al. (6) in order to detect possible mutagenicity of the described fat fractions, as well as the mono-, di-, tri- and tetrahydroxy octadecanoic and hydroperoxyoctadecadienoic acids as model substances.

EXPERIMENTAL

Test Substances

The polar fraction according to Guhr and Waibel (1) and the oxidized fatty acid fraction according to DGF standard method C-III 3 (77) (2) were obtained from processed deep frying fats from the local catering trade. These fractions were a generous gift of C. Gertz (Hagen, Germany). The model substances were: (a) 12-hydroxyoctadecanoic, and (b) 9,10-dihydroxyoctadecanoic acid (Serva, Heidelberg, Germany); (c) 9,10,12-trihydroxyoctadecanoic and (d) 9,10,12,13-tetrahydroxyoctadecanoic acid were produced by specific oxidation of the unsaturated fatty acids (7,8); (e) 9-hydroperoxyoctadeca-10,12-dienoic acid was obtained via enzymatic oxidation of linoleic acid according to Mathew et al. (9).

The solvents dimethyl sulfoxide and ethanol and the positive control substances, benzo(α)-pyrene, 2-aminoanthracene, 9-aminoacridine and sodium azide were commercially available (Merck, Darmstadt; Serva, Heidelberg, Germany).

Bacterial Strains

All bacterial tester strains were obtained from Professor B.N. Ames. We used the histidine auxotrophic strains of *Salmonella typhimurium* TA 1535 and TA 100 for the detection of base pair and TA 1537, TA 1538 and TA 98 for frameshift mutations. For each day's experiment, a fresh overnight culture was prepared containing $0.9\text{-}1.3 \cdot 10^9$ viable cells/ml. The tester strains were routinely checked for their genotypes (R-factor, uvrB deletion, rfa-mutation).

Assay Procedure

The Salmonella/microsome test was performed according to the original protocol of Ames et al. (6) and of de Serres and Shelby (10) with and without metabolic activation effected using rat liver homogenate. Rat liver homogenate was obtained from Aroclor-1254-induced Wistar rats. Fifty mg/plate of the centrifuged homogenate (S-9 fraction) were

used in the assay. The protein content was 4.4 mg/plate (11). The preincubation procedure was also performed according to Ames et al. (6). The test compound was preincubated for 30 min at 37 C with "S-9 mix" and the liquid suspension of the tester strain TA 100. The liquid medium assay was performed according to Mitchell (12) with *S. typhimurium* TA 100.

Description and Value of the Ames Test

The Ames test is a short-term mutagenicity test which uses various histidine-dependent *S. typhimurium* strains as indicator organisms. Under the influence of mutagenic substances, some bacteria revert back to the histidine nondependent wild type. These revertants are able to grow on a histidine-free minimal agar. The number of colonies which grow is counted and compared to a control showing the spontaneous mutation rate. The number of revertants/plate increases as the amount of mutagenic substance present increases. Such a response-relationship is of great significance when assessing mutagenicity of a compound. This relationship must be checked in all experiments using standard mutagens (13).

However, many substances are only effective mutagens after metabolic activation in mammals. The Ames assay is useful in that it allows one to simulate the mammalian metabolism. For this purpose, a liver fraction taken from rats is isolated which contains the mixed function oxidases and cytochrome $P_{448,450}$ along with soluble enzymes. This S-9 fraction is used with cofactors as metabolizing system (S-9 mix).

The value of the Ames test is the relatively good agreement between the data obtained in this test and the data obtained from carcinogenic experiments using animals. In several extensive studies designed to examine the ability of the Ames test to detect carcinogenic or noncarcinogenic chemicals in animal cancer tests, over 90% of the 200 carcinogens tested

TABLE I

Mutagenic Activity of Deep Frying Fat Fractions and Model Substances with *Salmonella typhimurium* in the Ames Assay

Test substance	Concentration (μg/plate)	Revertants per plate (mean of 3 plates)									
		TA 1535		TA 100		TA 1537		TA 1538		TA 98	
		-S-9	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	+S-9	-S-9	+S-9
Control (EtOH)	$79 \cdot 10^3$	11	31	106	105	19	9	16	45	24	51
Control (DMSO)	$110 \cdot 10^3$	10	26	107	110	20	14	17	38	26	51
Benzo(α)pyrene	1				387		52		79		108
	2				1007		127		152		192
	5				1610		183		344		410
	7				1600		139		272		599
NH_2-antracene	0.25		47								
	0.5		89								
	1		203								
NH_2-acridine	10					40					
	20					93					
NaN_3	5	1277									
	10	2575									
	20	4250									
Oxidized fatty acid fraction	500									34	58
	1000	10	23	94	99	14	10	14	34	29	48
	2500	11	18	120	100	11	9	13	21	26	27
	5000	6	19	101	100	10	6	13	17	20	16
Polar fraction	500									26	60
	1000	7	22	112	125	16	7	13	56	28	61
	2500	13	22	109	120	17	6	15	49	31	55
	5000	11	23	108	130	17	10	11	46	28	56
18:0-OH	500	10	21	106	113	15	4	15	34	24	47
	1000	10	13	107	101	15	4	14	20	21	51
	2500[a]	10	10	71	41[b]	18	5	19	13	14	41
18:0-$(OH)_2$	500	13	30	90	99	17	9	12	47	28	54
	1000	10	29	71	78	15	14	16	40	40	63
	2500[a]	12	21	9[b]	15[b]	15	8	19	46	25	60
18:0-$(OH)_3$	500	12	32	105	138	15	9	14	52	16	56
	1000	8	17	102	142	18	15	12	36	23	58
	2500	12	17	108	129[c]	17	11	16	44	19	54
28:0-$(OH)_4$	500	11	24	111	116	17	13	17	55	22	58
	1000	10	15	110	145	12	12	9	45	17	59
	2500	12	19	96	154[c]	14	15	10	51	19	51
18:2-OOH	10	11	31	100	124	9	10	22	48	19	45
	50	12	37	124	130	13	9	15	40	29	44
	100	4	21	104	70[c]	7	5	3[c]	12[c]	34	39
	250	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	31	38
	500	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]

[a]Precipitation of the test substance.

[b]Beginning of toxicity.

[c]Evidence of toxicity, apparent as thinning of bacterial background lawn.

TABLE II

Results Obtained with the Liquid Culture Test According to Mitchell with *Salmonella typhimurium* TA 100

Test substance	Amount (μg)	Revertants per plate (mean of 3 plates)	Survivors X 10^9 per plate (mean of 2 plates)	Corr. mutation rate X 10^9
Control (DMSO)	$100 \cdot 10^3$	80	4.2	
Benzo(α)pyrene	10	660	4.6	128
	20	900	1.1	752
18:0-$(OH)_3$	4000	50	4.9	-6
	10000	50	4.8	-6
	14000	60	3.4	-6
	20000	40	2.2	-18
18:0-$(OH)_4$	3300	120	6.6	6
	4600	90	5.6	2
	6600	100	5.4	4

were positive (13-18). In contrast to carcinogenic experiments with animals which are costly and time-consuming, the Ames test is relatively easy and inexpensive to perform. Because of the difference between human and bacterial cells, such tests are suitable only as preliminary tests.

RESULTS AND DISCUSSION

Table I shows the results obtained in the Ames assay using deep frying fat fractions and model test substances. All tests were performed in parallel with (+ S-9) and without (- S-9) metabolic activation; 100 μl of each of the 2 solvents dimethyl sulfoxide (DMSO) and ethanol (EtOH) were used as control plates to show the spontaneous mutation rates. From these results, the spontaneous mutation rates differ according to the tester strain used. A sample usually is considered mutagenic if it brings about a 2-fold or greater increase in the mutation rate. In order to assure the test system was working properly, especially with regard to the activity of the S-9 preparation, positive mutagenic substances were run simultaneously in the assays. The test range is limited (a) by precipitation of the test substance, and (b) by the dose, which is toxic to the test bacteria. Toxic effects can be seen when the number of spontaneous revertants diminishes, followed by the disappearance of the background lawn of nonrevertant bacteria. These toxic effects are well demonstrated with the hydroperoxide of linoleic acid as shown in Table I. In contrast to the standard mutagens, it can be seen that all the tested substances do not increase the mutation rates significantly. There is only a slight increase observed with tri- and tetrahydroxyoctadecanoic acid and the tester strain TA 100. However, the doubling of the spontaneous mutation rate is not reached. The conclusion drawn from the results summarized in Table I is that no mutagenic effect is observed with the tested samples using the Salmonella/microsome test according to Ames et al.

A modification of the Ames assay described was designed to increase its sensitivity to weak mutagens (6). The sample was preincubated for 30 min at 37 C with the S-9 mix and a liquid suspension of the tester strain. This preincubation effects a longer contact between substance, metabolites and the bacteria. We performed the preincubation procedure with the most sensitive tester strain, TA 100, and the 2 deep frying fat fractions as well as the 9-hydroperoxyoctadeca-10,12-dienoic acid. Once more, there was no mutagenic effect.

Another possibility to enhance the sensitivity of the bacterial short-term mutagenicity assays is the liquid culture test. This type of assay was performed according to Mitchell (12). In this assay, the test substance and metabolites formed by the S-9 mix were incubated with the tester strain in a liquid medium. During the first hr, the bacteria were held in a minimal medium; for the 3 hr that followed, they were allowed to grow in a nutrient broth. Afterwards, the bacteria were plated out onto a minimal agar to find the number of revertants and onto a nutrient agar to estimate the number of survivors. In this way, the difficulties incurred by toxic effects are eliminated. Table II shows a summary of the results obtained with the liquid culture test using *S. typhimurium* TA 100 and tri- and tetrahydroxyoctadecanoic acid. These 2 compounds were selected because of a slight increase in the mutation rates with TA 100, which was observed at the highest concentration possible using the Ames assay. An increase in the amount of the test compounds used was not

possible because of their toxic effects on the tester strain. The results of the liquid culture test reported in Table II were expressed as corrected mutation rates:

$$\text{Corr. mutation rate} = \frac{\text{Revertants Test} - \text{Revertants Control}}{\text{Survivors}}$$

In this test, a substance is considered to be mutagenic if a dose-response relationship is observed as can be seen with benzo(α)pyrene. The results of the tested hydroxy fatty acids only show corrected mutation rates around zero. It can therefore be concluded that no mutagenic effects are detectable up to the highest concentration that can be dissolved under test conditions.

To summarize our results,we were able to demonstrate that the deep frying fat fractions as well as the hydroxy fatty acids and the hydroperoxides used as model substances were not mutagenic in various modifications of the Salmonella/microsome assay. However, it should be emphasized that further investigations with test systems other than bacteria are necessary to come to a definite decision regarding the mutagenicity of these substances.

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Origins of the Cholesterol in Milk

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ABSTRACT

Studies were conducted to investigate the origin of milk cholesterol in the ruminant. In the first experiment, [1-^{14}C] sodium acetate was infused into one side of the udder of a lactating goat via the teat canal whereas in the second, [1,2-^{3}H] cholesterol was injected intravenously and concurrently with a [^{14}C] acetate intramammary infusion. In both experiments, blood and milk samples were collected at intervals for 6 days postinjection. Maximum unesterified cholesterol specific activity (sp act) in whole milk appeared at 78 hr after intravenous injections of ^{3}H cholesterol and within 3-7 hr after infusion of [^{14}C] acetate. Virtually all the tritium in milk was associated with unesterified cholesterol. The sp act of ^{14}C-labeled cholesterol was only 20% of gland-synthesized decanoic acid. Decanoic acid is known to be completely synthesized in the mammary gland, and, like cholesterol, acetate is its precursor. The results indicate that, although some milk cholesterol is synthesized in the mammary gland, it is derived principally from serum cholesterol. The data show also that serum cholesterol equilibrates with membrane cholesterol of the lactating cell prior to its secretion in milk.

INTRODUCTION

Although cholesterol is present in all mammalian milks, its origins are not well established. Cholesterol and its esters are associated primarily with milk fat globules (1,2); however, 20% of the total cholesterol is in the skim milk (2,3). Milk cholesterol may be derived by transfer from blood or from de novo synthesis from acetate within the mammary gland (4-13). Studies on the relative contribution of blood serum cholesterol and mammary gland-synthesized cholesterol to milk of the goat, as well as other species, have involved either oral or intravenous administration of radioactive precursors to the animals (5,6,9,14,15). Raphael et al. (12) used an abomasal injection of [^{14}C]-cholesterol to study transport of dietary cholesterol into goat milk. However, the relationship between the transfer of blood cholesterol into milk and the capacity of ruminant mammary tissue to synthesize milk cholesterol has never been evaluated in vivo.

MATERIALS AND METHODS

Two mid-lactation goats (1500 ml milk/day) from the University herd were held in metabolism stalls and fed a usual diet of hay, grain and water.

Experiment 1

After completely milking both sides of the udder, 250 μCi of [1,2-^{14}C] sodium acetate (53.5 mCi/nmol; New England Nuclear Corp., Boston, MA) in 10 ml of 0.9% NaCl were infused via the teat canal into the left mammary gland. Milk was collected at 3, 7 and 12 hr postinfusion, then every 12 hr up to 144 hr. After 144 hr, the radioactivity measurements of the activities in the milk were too low for useful analysis. Milk samples from the left and right glands were kept separate. Concurrently with milking, 10-ml blood samples were taken from a cathether in the right jugular vein.

Experiment 2

This experiment was similar to experiment 1 except that 6 hr before [^{14}C] acetate was infused into the mammary gland, a colloidal suspension of [1,2-^{3}H] cholesterol (250 μCi, 44.0 Ci/nmol; New England Nuclear Corp.) was injected into the jugular vein via the implanted catheter. Colloidal cholesterol was prepared by dissolving 0.25 mg of unlabeled cholesterol with the tracer cholesterol in 0.25 ml of 95% ethanol and adding 10 ml of 0.9% NaCl (16). The goat was milked dry 6 hr after the [^{3}H] cholesterol injection, which was immediately followed by mammary gland infusion of the [^{14}C] acetate. Blood and milk samples were taken at 1, 2, 4 and 6 hr after injection of tritiated cholesterol and at the previously designated milking times relative to acetate infusion.

Fractionation Procedures

Milk samples collected from the infused and noninfused mammary glands were treated in an identical manner. Portions (10-ml) of the uncooled whole milk samples were saved for lipid analyses. Other aliquots of uncooled milk samples were separated promptly into skim milk and a compacted layer of fat globules by centrifugation in 50-ml tubes at 1085 x g for 20 min at ambient temperature. Skim milks were decanted from under the fat globule

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layers.

All solvents were redistilled in glass. Total lipids were extracted from the whole milks, skim milks and globule fractions by the Roese-Gottlieb procedure (17). Solvents were removed from the lipid extracts with a rotary evaporator at 40 C. Lipids from 5 ml of blood serum were extracted according to the Folch et al. method (18). The solvent was removed under nitrogen.

Cholesterol Analysis

Unesterified cholesterol in the lipid samples was quantitatively isolated using digitonin/Celite columns as described by Schwartz et al. (19) and as modified by Witte (20). Modifications included reduction of sample size to ca. 0.5 g and addition of 1.0 mg of stigmasterol as an internal standard. Cholesterol was quantified using a Hewlett Packard 5750 gas chromatograph with a 6 ft × 1/8 in. glass column packed with 3% SP-2100 and 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). Separation was effected at 265 C and 40 ml/min helium carrier gas flow rate, with injector and detector temperatures at 300 C. Calculation of the absolute weight of cholesterol was performed by an internal standardization technique (21) and by comparison to a standard curve.

Decanoic Acid Specific Activity

The specific activity (sp act) of decanoic acid, a fatty acid synthesized from acetate almost exclusively within the gland (22), was determined for the whole milk samples from the infused and noninfused sides. Triglycerides from the hexane/benzene fraction of the cholesterol isolations were purified by thin layer chromatography (TLC) on silica gel plates (E. Merck, Darmstadt, Germany), using a petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) solvent system. The area corresponding to triglycerides following visualization by brief exposure to iodine vapors was scraped from the plate and the triglycerides eluted from the silica gel with chloroform.

Triglyceride fatty acids were methylated by transesterification with methanol containing boron trifluoride (23). Known volumes of the methyl ester extracts were chromatographed on a Hewlett Packard 5750 gas chromatograph equipped with a 6 ft × 1/8 in. stainless steel column packed with 15% DEGS on acid-washed Chromosorb W (Supelco, Inc.). Column temperature was programmed from 80 C to 175 C at 4 C/min with the helium carrier gas flow rate at 36 ml/min. Peak area of methyl decanoate was measured and the ester was quantified by comparison to areas on a standard curve for methyl decanoate (Applied Science Division, Milton Roy Co. Lab. Group, State College, PA).

Radioactive methyl decanoate was isolated by trapping the column effluent (from a heated outlet (245 C) in a glass U-tube filled with sand) during the retention time determined with standard methyl decanoate. Traps were maintained in a Dry Ice/acetone bath and had a recovery efficiency of 80%. Methyl decanoate trapped on the sand was eluted into a scintillation vial with 3 5-ml portions of scintillation fluid.

Cholesteryl esters were isolated by TLC as previously described for triglycerides. After elution, the cholesteryl esters were saponified by adding 2 ml of 0.5 N NaOH in methanol to the sample and refluxing for 10 min. Following addition of 2 ml of 6 N HCl, fatty acids and cholesterol were extracted with petroleum ether/diethyl ether (1:1, v/v). Cholesterol was separated from fatty acids by the McCarthy and Duthie method (24) and quantified by gas chromatography as described previously.

Radioactivity Measurements

Radioactivity measurements were performed with a Packard TriCarb Liquid Scintillation Spectrometer (Model 3330, Packard Instrument Co., Inc., Downers Grove, IL). Samples were counted in 10 ml of Quantafluor Scintillar (Mallinckrodt, Inc., St. Louis, MO) unless otherwise noted. Counting times were established on the basis of 1% counting error. In experiment 2, ^{3}H and ^{14}C were assayed simultaneously.

RESULTS

Specific activity of unesterified cholesterol from whole milk obtained from the infused and noninfused sides of the udder during the first 24 hr following infusion of [1,2-^{14}C] sodium acetate in experiment 1 are shown in Table I. Cholesterol sp act from the infused side were much higher than those from the noninfused side. This also occurred in experiment 2 (data not shown). About 0.1% of the ^{14}C appeared in milk cholesterol 3 hr after the [^{14}C] acetate infusion. Maximal sp act in milk cholesterol occurred within 7 hr postinfusion, followed by a slow, steady decline.

Cholesterol sp act values [^{3}H, ^{14}C] in whole milk from experiment 2 are presented in Figure 1. Specific activities for [^{14}C] cholesterol rose rapidly after [^{14}C] acetate infusion followed by a gradual decline. Tritiated cholesterol increased initially and reached a plateau of maximal sp act at 78 hr after intravenous

TABLE I

Specific Activity of Unesterified Cholesterol in Whole Milk following Infusion of [1,2-^{14}C]-Sodium Acetate (Experiment 1)

Postinfusion time (hr)	Milk Unesterified cholesterol	
	Infused gland	Noninfused gland
	dpm/mg	
3	3366	22
7	3558	0
12	2891	0
24	2554	499

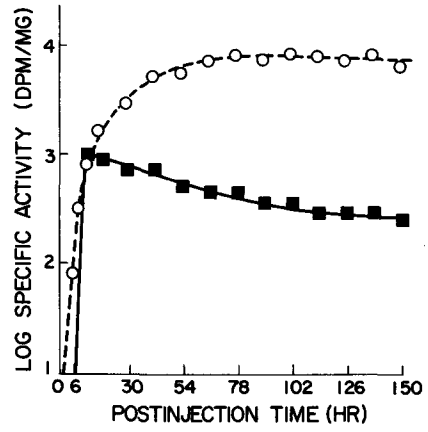

FIG. 1. Specific activity of unesterified cholesterol in whole milk following intravenous injection of [1,2-^{3}H] cholesterol (○—○) and intramammary infusion of [1,2-^{14}C] sodium acetate (■—■). Acetate was infused into the mammary gland via a teat canal 6 hr after injection of cholesterol.

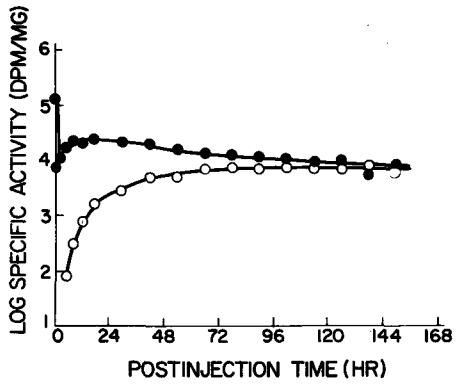

FIG. 2. Specific activity of unesterified cholesterol in blood serum (●—●) and whole milk (○—○) following intravenous injection of [1,2-^{3}H] cholesterol.

injection of [^{3}H] cholesterol. This data is similar to that reported for the guinea pig (5), rat (7) and goat (11).

Specific activity of tritiated cholesterol in blood and milk over time is given in Figure 2. After a sharp decline within the first 2 hr, the sp act of blood serum cholesterol gradually increased 4-12 hr postinjection after which it decreased very gradually. On the other hand, ^{3}H cholesterol activity in whole milk rose for 42 hr following intravenous injection of tritiated cholesterol. After 78 hr, milk cholesterol activity leveled and by the sixth day it approached the serum sp act values.

The concentration of cholesteryl esters in whole milk was ca. 5% of the total cholesterol, which agrees with literature values (12). Total lipid extracts and unesterified cholesterol fractions isolated from 10 ml of whole milk had virtually identical ^{3}H activity values in samples from 13- through 150-hr postinjection. At no time did cholesterol activity fall below 90% of the total lipid activity and in 8 out of 13 samples it was 100%. Closeness of the activity values for the 2 fractions indicates that labeling of the milk cholesterol ester fraction by the tritiated cholesterol was extremely limited. Following [^{14}C] acetate infusion (experiment 2), label was incorporated only into the fatty acid moiety of milk cholesterol esters.

Figure 3 shows the activity from [^{14}C]-acetate which was incorporated into milk cholesterol and decanoic acid (experiment 1). This comparison may give an indication of the cholesterol-synthesizing capacity of the mammary gland since decanoic acid is only syn-

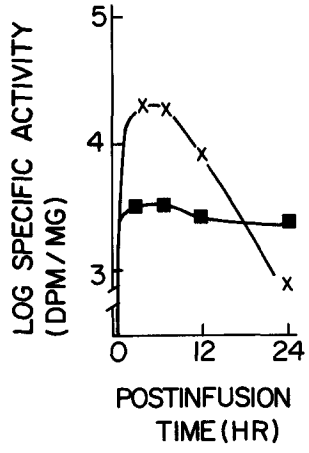

FIG. 3. Specific activities of cholesterol (■—■) and decanoic acid (X—X) synthesized by the mammary gland following intramammary infusion of [1,2-^{14}C] sodium acetate (experiment 1).

thesized in the mammary gland (22) and cholesterol and fatty acids are secreted into milk in a fairly constant ratio. Because the synthesis cycle of fat globules in the goat is 4-6 hr (25), the best time to make a comparison is at the plateau area between 3 and 12 hr post-infusion. During that time interval, sp act of cholesterol was found to be diluted by ca. 80% compared to the decanoic acid activity. This suggests that ca. 20% of the milk cholesterol is synthesized from acetate in the mammary gland and 80% is derived from the blood.

DISCUSSION

The results of this study confirm that there are 2 sources of milk cholesterol, namely, the blood and de novo synthesis in the mammary gland (5,6,9). The criterion by which to decide whether [1,2-^{14}C] acetate was used by mammary tissue for synthesis of milk cholesterol rests on a comparison of the sp act of cholesterol in milk samples isolated from the infused and noninfused mammary glands. If the sp act of the cholesterol from the infused side was greater than that from the noninfused side, the cholesterol was synthesized primarily by the gland. If the radioactive acetate was absorbed from the infused gland and the cholesterol synthesized in another tissue of the body, it would be transported by the blood equally to both glands. This is based on the evidence that there is no exchange or diffusion of substances between halves of the udder (26,27).

As a result of the large amount of acetate production in the rumen and its subsequent mobilization in blood to various body tissues, including the mammary gland for milk fat synthesis, it is likely that dilution of radioactive acetate with unlabeled acetate took place. This, in turn, could cause the sp act values for [^{14}C] cholesterol in milk from the noninfused side of the mammary gland to be very low (Table I). The decidely greater amount of [^{14}C] acetate incorporated into milk cholesterol from the infused side of the udder indicates that some milk cholesterol is synthesized de novo in the mammary gland. Although the [^{14}C] acetate infusion experiments alone do not lend themselves to any quantitative interpretation, concurrent study of [^{14}C] acetate incorporation into decanoic acid suggests that ca. 80% of milk cholesterol is of blood origin. Cholesterol-feeding experiments with goats conducted by Mills et al. (9) have shown that a smaller amount of milk cholesterol, 50-60%, is derived from blood. However, since serum cholesterol is readily transported to the mammary gland (12), it seems this would obviate the need for extensive glandular de novo synthesis.

The rapid initial disappearance of serum [^{3}H] cholesterol followed by reappearance in blood a short time later (Fig. 2) agrees with observations for the goat (12), rat (28) and human (29). An increased appearance of labeled cholesteryl esters in blood serum occurred in this study which is similar to observations described by Nilsson and Zilversmit (28). This phenomenon indicates active lecithin:cholesterol acyltransferase (LCAT) activity in plasma of the lactating goat. The very low amount of tritium label in whole milk cholesterol compared to blood serum shortly following intravenous injection of [^{3}H] cholesterol (Fig. 2) implies that a negligible amount of injected cholesterol traveled directly to the mammary gland. The bulk of injected cholesterol was cleared by the liver and incorporated into serum lipoproteins prior to uptake by the mammary gland (29).

Little is known about the involvement of very low density, low density and high density lipoproteins for cholesterol transport into mammary cells of the ruminant (30,31). Even transport of dietary cholesterol to mammary gland by chylomicrons, which has been extensively investigated in the rat (5), is an unsettled issue for ruminants (30). Lipids are transported to peripheral tissues via serum protein carriers. Phillippy and McCarthy (32) reported that maximal serum albumin sp act appeared in milk 12 hr after [^{125}I] serum albumin was injected intravenously into a goat. The rapid peak appearance of radioactive serum albumin in milk reported by these scientists compared with the much longer and more gradual increase of serum [^{3}H] cholesterol to a plateau in milk found in our study (Fig. 4) implies that cholesterol is not transported into milk in the form of a serum protein-cholesterol complex such as a lipoprotein. The lack of any tritium in milk cholesterol esters (see preceding) also supports this contention. Rather, serum-derived cholesterol apparently equilibrates throughout mammary cell membranes prior to its transfer into milk. A similar conclusion was drawn by Easter (8) in studies of serum cholesterol transport into rat milk. The persistent release of cholesterol synthesized in the mammary tissue from acetate into milk over a 150-hr period (Fig. 1) also implies an origin of milk cholesterol in the membrane pool of the lactating cell.

The closeness of the total and unesterified cholesterol activities for ^{3}H labeling makes it

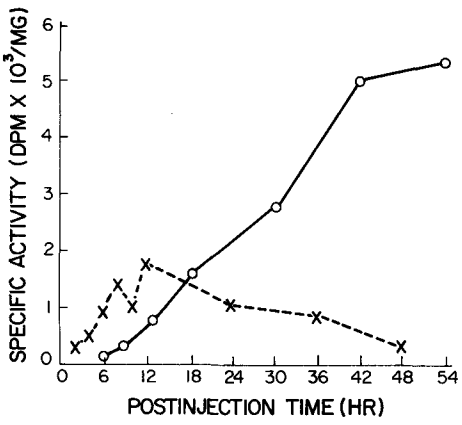

FIG. 4. Comparison of the transfer of cholesterol (o—o) and serum albumin (X—X) from blood into milk following intravenous injection of [1,2-^{3}H]-cholesterol or [^{125}I] serum albumin into the lactating goat (serum albumin data taken from ref. 32).

apparent that virtually all of the cholesterol which enters milk intact from the bloodstream is in the unesterified form. The ultimate coincidence in sp act of serum and milk unesterified cholesterol (Fig. 2) also is indicative of this possibility. Absence of any ^{3}H activity in the cholesterol esters of milk, even though measured only in the initial 48-hr sample collection period, is puzzling and does not concur with data previously reported. After abomasal injection of [^{14}C] cholesterol, Raphael et al. (12) had observed a significant and unique cyclic labeling pattern of milk cholesteryl esters in goats. A contribution to serum cholesterol metabolism by intestinal mucosa may account for this discrepancy.

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Experimental Nephrotic Syndrome in the Rat Induced by Puromycin Aminonucleoside: Hepatic Synthesis of Lipoproteins and Apolipoproteins[1]

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ABSTRACT

Hepatic synthesis of lipoproteins and apolipoproteins was investigated in male Wistar rats with severe nephrotic syndrome induced by puromycin aminonucleoside by incubating liver slices with a mixture of ^{14}C-amino acids. Labeled lipoproteins were separated by preparative ultracentrifugation from the incubation medium after the addition of carrier plasma. The incorporation of ^{14}C-amino acids into very low density lipoproteins (VLDL) (1.006 g/ml), low density lipoproteins (LDL) (1.006-1.063 g/ml) and high density lipoproteins (HDL) (1.063-1.210 g/ml) was increased in nephrotic liver 6.1-, 5.7- and 5.0-fold, respectively. The measurement of radioactivity associated to apolipoproteins isolated by SDS-PAGE documented an increased incorporation into apolipoprotein E (apoE) of nephrotic VLDL (33.1% vs 20% of the total radioactivity incorporated into VLDL apoproteins) and a markedly increased incorporation into apolipoprotein A-I (apoA-I) of nephrotic HDL (44.3% vs 16.3% of the total radioactivity incorporated into HDL apoproteins). In nephrotic liver, the total incorporation of amino acids into apolipoproteins (apoVLDL + apoLDL + apoHDL) was increased 12.6 times for apoA-I, 6.4 times for apoB, 5.0 times for apoE, 4.2 times for apoC + apoA-II and 2.5 times for apoA-IV. We suggest that, in nephrotic liver: (a) the synthesis of VLDL, LDL and HDL is increased, and (b) the total synthesis of apoA-I is selectively incresed when compared to that of the other apolipoproteins.

INTRODUCTION

Although it has been suggested for several years that an increased hepatic synthesis of plasma lipoproteins may be the crucial factor in the origin of both human (1,2) and experimental (3-6) nephrotic hyperlipoproteinemia, few studies have focused on the hepatic synthesis of lipoproteins in nephrotic animals (7-10) and the actual role of the hepatic overproduction of lipoproteins in the pathogenesis of nephrotic hyperlipoproteinemia is not yet firmly established. Furthermore, the question can be raised as to whether the increased synthesis of lipoproteins which is thought to occur in nephrotic liver is confined to some classes of plasma lipoproteins or involves all lipoprotein species as a nonspecific response to the loss of plasma protein in the urine.

The involvement of all lipoprotein species is particularly interesting since we recently found (5) that, in nephrotic rats, the concentration of all plasma lipoproteins is markedly elevated and their apoprotein composition is abnormal—apolipoprotein E (apoE) is elevated in both very low density lipoproteins (VLDL) and low density lipoproteins (LDL); apolipoprotein A-I (apoA-I) is present in LDL; apoA-I is increased and apolipoprotein A-IV (apoA-IV) and apolipoprotein (apoE) are diminished in high density lipoproteins (HDL).

Since it is unknown whether these changes result from the hepatic overproduction of abnormal lipoprotein particles or whether they represent the result of an altered intravascular or cellular metabolism of plasma lipoproteins, we have undertaken this study in an attempt to answer the following questions: (a) whether the synthesis of some lipoproteins and/or apolipoproteins was selectively stimulated in nephrotic liver and (b) to what extent the hepatic overproduction of plasma lipoproteins could account for the changes of concentration and apoprotein composition of lipoproteins of nephrotic rat plasma.

MATERIALS AND METHODS

Materials

Puromycin aminonucleoside (6-dimethylamino[3′-amino-3′-deoxyribosyl]purine) was obtained from Sigma Chemical Co. (St. Louis, MO). Eagle's basal medium was obtained from Wellcome Research Laboratories (Beckenham, England). The mixture of ^{14}C-amino acids was purchased from New England Nuclear (Dreieich, West Germany). Instagel was from Pack-

[1] Preliminary reports of this work were presented at the Annual Meeting of the European Society for the Study of the Liver (Düsseldorf, September 13-15, 1979); at the 5th International Symposium on Atherosclerosis (Houston, November 6-9, 1979) and at the Annual Meeting of the Italian Society for the Study of the Liver (Rome, December 14-15, 1979).

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ard Instrument Co. (Downers Grove, IL). Other reagents were obtained from C. Erba (Milan, Italy).

Animals

Twenty-four male Wistar rats (285-300 g in weight) were used in this study. Twelve rats were made nephrotic by 7 injections of puromycin aminonucleoside (20 mg/kg) over a period of 7 days and 12 rats were used as controls. Food intake was restricted in the control rats at the mean value of food consumption of the nephrotic group. The development of the disease was monitored by daily measurement of the protein loss in the urine (5). The animals used in this study were the same as those employed in a previous study from this laboratory (11).

Blood Samples

Seven rats from each group were used as blood donors. Five days after the withdrawal of the chemical, they were bled from the carotid artery. K_3EDTA (1.3 g/l) was used as anticoagulant. Plasma was separated, pooled and stored at 4 C for 24 hr before use.

Incubation of Liver Slices

Five nephrotic and 5 control rats were killed by decapitation and livers were rapidly removed and washed in cold 0.154 M NaCl, pH 7.4. Liver slices (117-168 mg) were prepared from each animal in duplicate and incubated in 2.5 ml of Eagle's basal medium containing 0.5 mM bovine serum albumin and 0.5 mM palmitic acid. Each flask contained 4 μCi of a mixture of ^{14}C-amino acids. The incubations were carried out in a Dubnhoff metabolic shaker at 125 oscillations/min at 37 C under an atmosphere of O_2/CO_2, 95:5 for 2 hr. At the end of the incubation, the medium was collected and centrifuged at 600 g for 10 min in order to remove particulate material. The supernatants of the liver slices of the animals of each group were pooled together before the addition of carrier plasma. Plasma from control rats was added to the pooled supernatants of control liver slices, whereas plasma of nephrotic rats was added to the pooled supernatants of nephrotic liver slices. These supernatants were subsequently dialyzed against NaCl 0.154 M containing EDTA 10^{-3} M, pH 7.4, until radioactivity of the dialysis buffer reached negligible values.

Liver slices were washed 3 times with dialysis buffer, homogenized in 2 ml of NaCl (0.154 M) and stored at -20 C for 3-4 weeks before the measurement of intracellular protein synthesis.

Measurement of Intracellular and Extracellular Protein Synthesis

Aqueous trichloroacetic acid was added to an aliquot of homogenated liver slices or of incubation medium to a final concentration of 50 g/l. Protein was sedimented at 1,500 g for 10 min and the supernatant discarded. The pellet was then washed 3 times with trichloroacetic acid (50 g/l) to remove residual trichloroacetic acid soluble radioactivity. After the third washing, the amount of radioactivity in the trichloroacetic acid soluble fraction was usually less than 3-4% of the radioactivity associated with the pellet. The pellet was finally dissolved in 1 ml of 0.1 N NaOH and transferred into a scintillation vial.

Samples were counted in a Packard C 2425 Tri Carb liquid scintillation spectrometer after the addition of 15 ml of Istagel.

Separation of Lipoproteins from the Supernatant of Liver Slices

An aliquot of supernatant supplemented with carrier plasma was taken for the separation of plasma lipoproteins by preparative ultracentrifugation. With the addition of appropriate carrier plasma prior to ultracentrifugation, we assumed that the floating properties of the newly secreted lipoproteins were similar to those of lipoproteins circulating in plasma. VLDL (1.006 g/ml), LDL (1.006-1.063 g/ml) and HDL (1.063-1.210 g/ml) were separated in a Beckman $L_{5_{65}}$ ultracentrifuge using a 50 titanium rotor at 4 C as reported previously (5). No attempt was made to quantify the radioactivity associated with lipoproteins and/or apolipoproteins which were present in the 1.210 ml infranate.

Measurement of Lipoprotein Synthesis

Bovine serum albumin (1 mg) was added to aliquots of each lipoprotein fraction which were then precipitated by trichloroacetic acid at a final concentration of 50 g/l. Samples were further processed as described for the measurement of intracellular and extracellular protein synthesis.

Separation of Apolipoproteins by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Measurement of Radioactivity Associated to Isolated Apolipoproteins

Lipoprotein protein was measured according to Lowry et al. (12). Small aliquots of lipoprotein solution were heated at 100 C for 2 min after the addition of 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol. Fifty μg of protein were then applied to 10% polyacrylamide gels

containing sodium dodecyl sulfate (13). The protein bands corresponding to apolipoprotein B (apoB) (origin of the gel), apoA-IV (MW = 44-46,000), ApoE (MW = 33-35,000), apoA-I (26-28,000) and apolipoprotein C (apoC) + reduced apolipoprotein A-II (apoA-II) (MW = 6-12,000) were cut and transferred into glass scintillation vials. After the addition of 0.2 ml 32% H_2O_2, the vials were incubated at 50-60 C for 8-10 hr until the solution was completely clear. One ml of distilled water was then added and the content of the vials was flushed with N_2 in order to remove dissolved O_2.

Instagel (15 ml) was then added and the samples counted after normal light and temperature adaptation. Blank samples containing unlabeled apoproteins previously separated by SDS-PAGE were prepared as described for the labeled samples and the resulting counts were subtracted in order to eliminate spurious counts.

Measurement of Radioactivity Associated to Albumin

Aliquots of ultracentrifugal residue (> 1.210 g/ml) obtained from supernatants of both control and nephrotic liver slices were processed as described for the measurement of intracellular and extracellular protein synthesis. After 3 washings in trichloroacetic acid (50 g/l), the pellet was suspended in trichloroacetic acid (50 g/l) in 75% ethanol. Protein was sedimented at 1,500 g for 10 min and radioactivity was measured in the trichloroacetic acid soluble fraction (14).

Statistical Methods

Data obtained from single animals within each group were expressed as the mean ± standard deviation. The difference between groups was calculated using Student's t-test. When the data were obtained from pooled material, they were expressed as the mean of duplicate determinations.

RESULTS

Synthesis of Intracellular and Extracellular Proteins

The incorporation of ^{14}C-aminoacids into intracellular and extracellular proteins by control and nephrotic liver is shown in Figure 1. In nephrotic rats, the incorporation of ^{14}C-amino acids into intracellular and extracellular proteins was 2.1- and 2.6-fold, respectively, of the corresponding value found in the control rats.

Synthesis of Lipoproteins and Albumin

The ^{14}C-amino acid incorporation into plasma lipoproteins by control and nephrotic liver slices is shown in Figure 2. In nephrotic liver, the rate of incorporation of ^{14}C-amino acids into VLDL, LDL and HDL was 6.1-, 5.7- and 5.0-fold, respectively, of the values found for the corresponding lipoproteins secreted by control livers. The ^{14}C-amino acid incorporation into plasma albumin was 40,635 dpm/g/hr in nephrotic liver and 12,852 dpm/g/hr in the control liver.

Synthesis of Apolipoproteins

The percentage distribution of radioactivity among the various apoproteins separated by SDS-PAGE is shown in Table I. In nephrotic rats, the percentage of radioactivity associated to apoA-I of HDL was strikingly increased and so was, although to a lesser extent, the radioactivity found in apoE of VLDL. The relative incorporation of ^{14}C-amino acids into both

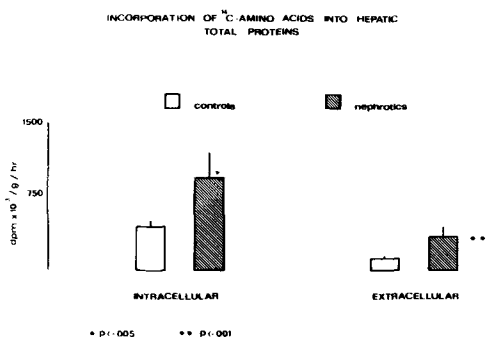

FIG. 1. Incorporation of ^{14}C-amino acids into intracellular and extracellular proteins by control and nephrotic liver slices. Data represent the mean ± standard deviation from 5 animals in each group.

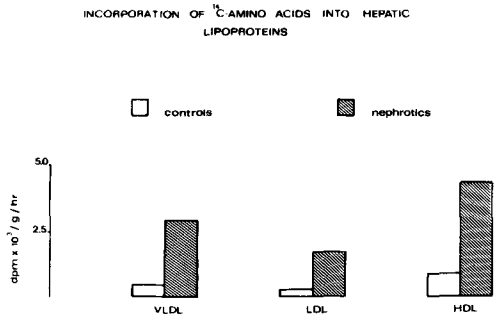

FIG. 2. Incorporation of ^{14}C-amino acids into plasma lipoproteins secreted by control and nephrotic liver slices. Data represent the values obtained after pooling the supernatants from 5 duplicate incubations of liver slices of each groups of rats.

apoA-IV and apoC + apoA-II apparently was reduced in all lipoprotein fractions. The total incorporation of ^{14}C-amino acids into the individual apolipoproteins was calculated from the data reported in Figure 2 and Table I. Table II shows that, in nephrotic rat liver: (a) the rate of incorporation of ^{14}C-amino acids into all apolipoproteins was increased, and (b) this increase was much higher in the apoA-I than in the other apolipoproteins.

DISCUSSION

In this study, we investigated the hepatic synthesis of serum lipoproteins and apolipoproteins in normal rats and in rats with a severe nephrotic syndrome induced by puromycin aminonucleoside. As an experimental approach, we employed liver slices incubated with ^{14}C-amino acids and measured the radioactivity in the lipoproteins and apolipoproteins secreted into the medium and separated by preparative ultracentrifugation after the addition of carrier plasma. In both control and nephrotic liver, the incorporation of ^{14}C-precursor occurred in all lipoprotein classes (Fig. 2). The major incorporation was found in HDL and, to a lesser extent, in VLDL. Surprisingly, however, a fairly high number of counts was found in the LDL fraction (Fig. 2); this observation apparently is in contrast to other studies which indicated that the incorporation of amino acids into serum lipoproteins secreted by rat liver was almost exclusively confined to VLDL (15). We interpret our results by assuming that, under our experimental conditions, there could have been a passive exchange of newly synthetized apolipoproteins between the lipoproteins secreted by the liver and those added as carriers. This interpretation is supported by the observation that, whenever carrier plasma was used in both in vivo studies (15) and isolated perfused liver (16), radioactivity persisted in the LDL fraction. Obviously, the possibility cannot be ruled out that rat liver is capable of secreting lipoproteins floating in the 1.006-1.050 g/ml density interval (LDL), as can be inferred from liver perfusion studies performed with a non-recirculating medium (17) and from kinetic data (18).

Our observations indicate that the ^{14}C-amino acid incorporation into serum lipoproteins secreted by nephrotic liver was 5-6-fold higher than the one found in the liver of control rats.

TABLE I

Percent Distribution of Radioactivity among Apolipoprotein Fractions

	ApoB	ApoA-IV	ApoE	ApoA-I	ApoC + ApoA-II
VLDL-C	26.3	10.4	20.0	5.2	29.3
VLDL-N	29.2	5.0	33.1	3.5	25.5
LDL-C	25.1	8.4	15.3	12.0	36.6
LDL-N	25.2	4.4	14.0	18.7	29.5
HDL-C	4.1	8.9	27.9	16.2	29.6
HDL-N	5.1	3.5	15.9	44.3	20.3

Percentage distribution of radioactivity among apolipoproteins separated by SDS-PAGE. VLDL-C, LDL-C and HDL-C indicate the corresponding lipoproteins from control rats whereas VLDL-N, LDL-N and HDL-N indicate the corresponding lipoproteins from nephrotic rats. Data represent values obtained after pooling the supernatants from 5 duplicate incubations of liver slices of each animal.

TABLE II

Total Incorporation of ^{14}C-Amino Acids into Apolipoproteins by Liver Slices (in dpm/g/hr)

	ApoA-I	ApoA-IV	ApoB	ApoC + ApoA-II	ApoE
Controls	193	157	244	526	397
Nephrotics	2435	388	1572	2220	1978
Nephrotics/ controls	12.6	2.5	6.4	4.2	5.0

Total incorporation of ^{14}C-amino acid into individual apolipoproteins by control and nephrotic liver slices. Data were calculated from those reported in Fig. 2 and Table I.

Interesting is this apparent increased synthesis of lipoproteins that was associated with an enhanced hepatic incorporation of ^{3}H-water and ^{3}H-palmitate into neutral lipids, as we reported in a previous communication (19). Thus, it is apparent that an increased hepatic lipogenesis may be one of the factors which promotes the synthesis of apolipoproteins in the liver of nephrotic animals. Taken together, our findings are in good agreement with previous studies carried out using liver slices (7), isolated and perfused rat liver (8) microsomes (9), which indicated that the hepatic synthesis of serum lipoproteins was increased in rats with nephrotic syndromes induced by antikidney serum. A further support to our data comes from a recent in vivo study (10) which demonstrated that, in rats with nephrotic syndrome induced by puromycin aminonucleoside, the incorporation of ^{14}C-leucine into 1.063 g/ml lipoproteins (VLDL + LDL) was increased 5.2 times and into 1.063 1.210 g/ml lipoproteins (HDL) 2.9 times.

We also observed that, in nephrotic rats, the relative incorporation of amino acids into apoE associated to VLDL and into apoA-I associated to HDL was increased (Table I). We think this finding may explain the increased apoE content of VLDL and the increased apoA-I content of HDL we observed in the plasma of nephrotic rats (5). Obviously, these conclusions should be taken with caution since we are aware that during the isolation of lipoproteins by preparative ultracentrifugation there may be selective loss of some apolipoproteins (e.g., apoE [19]). Since in this study we did not quantify the radioactivity present in any apoprotein of the d > 1.210 g/ml infranate, the actual incorporation of ^{14}C-amino acid into apoE and possibly into apoA-I might have been underestimated. With these limitations in mind, it may be notable that in nephrotic rats the total incorporation of amino acids into individual apolipoproteins was increased at different rates (Table II). The incorporation of amino acids into apoA-IV, apoC + apoA-II, apoE and apoB showed 2.5-, 4.2-, 5.0- and 6.4-fold increases, respectively, whereas the incorporation into apoA-I showed a 12.6-fold increase. Whichever the mechanism underlying these differences may be, the increased synthesis of apolipoproteins which occurs in nephrotic liver apparently is selective and concerns mainly the synthesis of apoA-I. In theory, it can be argued that our conclusion concerning the distribution of radioactivity in the various apoproteins is based on a not yet proven assumption, i.e., that the newly secreted apoproteins have an electrophoretic mobility in SDS-PAGE similar to that of the corresponding apoproteins associated to the lipoproteins circulating in plasma. We think that there is evidence indicating this assumption is valid at least for apoA-I and apoE. Felker et al. (20) observed that the mobility of these 2 apolipoproteins isolated from VLDL and HDL newly secreted by the isolated and perfused rat liver does superimpose to that of the corresponding apoproteins isolated from plasma VLDL and HDL.

Very recently, Marsh and Sparks (21) reported that liver of rats with nephrotic syndrome induced by puromycin aminonucleoside which had been perfused with a non-recirculating medium secreted 2-3 times the amount of lipoproteins produced by control liver. These authors further documented that apoA-I content of nascent HDL was increased from 16 to 52% in nephrotic rats and that the total secretion of apoA-I was 8.4-fold higher in nephrotic than in control liver. In addition, they found that the secretion of apoB, apoE and apoC was slightly increased (apoB and apoE) or unchanged (apoC). These data further support the results obtained in our study.

Our observations, as well as those of previous studies (7-10,21), would strongly suggest that the hepatic overproduction of serum lipoproteins may be responsible for the increased levels of lipoproteins found in nephrotic plasma (5). It seems likely, however, that other factors, such as a defect in the intravascular or cellular catabolism of some plasma lipoproteins, may be involved in the pathogenesis of nephrotic hyperlipoproteinemia.

ACKNOWLEDGMENT

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Distribution of Deuterium-labeled *cis*- and *trans*-12-Octadecenoic Acids in Human Plasma and Lipoprotein Lipids

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ABSTRACT

Triglycerides containing *cis*- and *trans*-12-octadecenoic acid (12*c*-18:1 and 12*t*-18:1) and *cis*-9-octadecenoic acid (9*c*-18:1) labeled with deuterium were fed to 2 young adult male subjects. These fatty isomers each contained a different number of deuterium labels, which allowed mass spectrometric analysis to distinguish among them after they were fed as a mixture. This approach results in a direct comparison of the absorption and distribution of these 3 monoenoic acids into blood plasma and lipoprotein lipids. Plasma lipid data indicated that all phospholipid fractions selectively incorporate 12*c*-18:1 and 12*t*-18:1 in preference to 9*c*-18:1. Discrimination against 12*c*-18:1 and 12*t*-18:1 compared to 9*c*-18:1 was found in the plasma neutral lipids, with a strong discrimination against 12*t*-18:1 incorporation into the cholesteryl ester fraction. Considerable reduction in the percentage of linoleic and arachidonic acid was observed when 12-18:1 isomers were incorporated in plasma triglyceride, phosphatidylcholine and sphingomyelin samples. Chylomicron lipid analyses indicated that all isomers were well absorbed. Variation was observed in the relative distribution of 12*c*-18:1, 12*t*-18:1 and 9*c*-18:1 between the very low density, low density and high density lipoprotein lipid classes. No desaturation of 12*c*-18:1 to linoleic acid was detected.

INTRODUCTION

Positional isomers of fatty acid are found in foods that contain partially hydrogenated vegetable fat (1,2). *cis*- and *trans*-Octadecenoic acid positional isomers occur in these oils and normally have the double bond distributed between the 7 through the 14 positions.

Various aspects of metabolic studies with individual *cis* and *trans* positional octadecenoic acid isomers have been reviewed recently by Lanser (3), Lands (4), Wood (5) and Holman (6). Studies using both animal and in vitro models have been used to characterize various effects and enzymatic reactions involving fatty acid isomers. Results of human experiments designed to measure the effect of partially hydrogenated vegetable oils have been reviewed by Emken (7).

Only one study using isotope-labeled fats has been reported that describes the in vivo absorption and distribution of a specific fatty acid isomer into various human neutral blood lipids (8) and phospholipids (9). In that study, the uptake of deuterium labeled *cis*- and *trans*-9-octadecenoic acids (9*c*-18:1 and 9*t*-18:1) by human plasma, erythrocyte and platelet neutral and phospholipids was followed. Definite differences were found in the relative degree of incorporation of 9*t*-18:1 compared to 9*c*-18:1 into various blood lipid fractions. This investigation is an extension of these studies and compares the absorption and distribution of *trans*-12-octadecenoic acid (12*t*-18:1) and *cis*-12-octadecenoic acid (12*c*-18:1) relative to 9*c*-18:1 in individual lipid classes isolated from human plasma, chylomicrons (CHYLO), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions.

EXPERIMENTAL

Triple-labeled Methodology

An experimental approach employing triple-labeled methodology was used. This technique is an extension of earlier dual-labeled methodology but involves feeding a mixture of 3 triglycerides. Each pure triglyceride contained one of the 3 differently labeled fatty acids (8-10). Triple-labeled methodology consists of labeling each of 3 different fatty acids with a different number of deuterium atoms. Thus, when the mixture is fed, each of the labeled fatty acids can be quantitatively measured by mass spectroscopic analysis. Unlabeled fatty acids do not interfere with the analysis. Since all the labeled fatty acids are fed at the same time, each subject serves as his own control and analytical errors are identical for each fatty acid. An additional advantage of triple-labeled studies over single-labeled studies is that variation resulting from dietary, genetic, or bio-

logical effects are reduced. This results in data that are more precise than if each labeled fatty acid were fed at different times to the same subject. Triple-labeled studies also allow 3 times more data to be obtained from each subject than do single-labeled studies.

Deuterated Fats Fed

Synthesis of deuterated fatty acids and their conversion to triglycerides have been described previously (11-13). Each triglyceride contained one deuterated fatty acid. The amount and identity of the labeled fats in the triglycerides fed to each subject are summarized in Table I. A different labeling pattern was used for each of the mixtures to reduce the possibility of a systematic error that would bias the data. The rationale for this precaution is similar to the reverse-labeling technique recommended for dual radioisotope experiments. If the isotopes influence the metabolism, then variation in the labeling pattern should produce a noticeable difference between data from similar experiments, and that data should follow a pattern related to the labeled fats.

Subjects, Sample Preparation and Sampling

The subjects were 2 Caucasian males, ages 28 and 26. They were in excellent health as judged by medical exminations and had no history of congenital ailments. Their weight, blood pressure, serum cholesterol and triglyceride levels were normal. The subjects were placed on a 1800-kcal diet normally prescribed for diabetics for 1 wk before the feeding study and did not eat for 10 hr before the feeding study. A normal lunch and evening meal were allowed the day the labeled fats were fed. The subjects were fed the mixture of deuterated triglycerides (TG) in place of their normal breakfast. The TG were emulsified with calcium caseinate (Sevar-tone® 100; 30 g), dextrose (30 g), sucrose (15 g) and water (200 ml). Emulsification was achieved by blending the caseinate and sugars with water heated to 60-65 C. The deuterated fat mixtures were heated to 60 C and slowly blended with the warm mixture of other materials. The final mixture had the texture and consistency of a milk shake, and it was cooled to 15 C before feeding. Blood samples were drawn at 0, 2, 4, 6, 8, 12, 15, 24 and 48 hr. Other details of the feeding and sampling procedures were similar to those described previously (8,9).

Analysis of Blood Lipids

Plasma lipids were isolated by previously described procedures (8,9). Preparative ultracentrifugation was used to isolate lipoprotein fractions from serum samples (14,15). The purity of the isolated lipoprotein fractions was verified by electrophoresis using the Pfizer Pol-E-Film® system. Previously described procedures were used to extract and fractionate the various lipid classes and convert the fatty acids from these individual lipid classes to their methyl esters (9,16-18). Gas chromatography-mass spectrometry (GC-MS) analysis of the deuterated fatty methyl ester derivatives was accomplished as reported elsewhere (19). The accuracy of the GC-MS data was estimated by analysis of weighed standard mixtures containing 9*c*-18:1-d_0, 9*c*-18:1-d_2, and 9*t*-18:1-d_4. Standard deviations of ±0.3% were obtained for those standards representative of the samples encountered in this study. Acyl position analysis of phosphatidylcholine (PC) was accomplished using phospholipase A_2 from *Ophiophagus hannah* venom (20).

Calculation of Selectivity Factors

Selectivity values for 12*t*-18:1 and 12*c*-18:1

TABLE I

Deuterated Fatty Acids in Triglycerides Used in Human Studies[a]

Subject	Mixture fed	Total weight (g)	Ratio in fed mixture		
			12*t*/9*c*	12*c*/9*c*	12*t*/12*c*
1	9*c*-18:1-9,10-d_2 12*c*-18:1-9,10,15,15,16,16-d_6 12*t*-18:1-15,15,16,16-d_4	28.5	0.97	1.05	0.98
2	9*c*-18:1-14,14,15,15,17,18-d_6 12*c*-18:1-15,15,16,16-d_4 12*t*-18:1-9,10-d_2	28.8	1.10	1.12	0.98

[a]Abbreviations: Geneva numbering system used. The first number indicates the position of the double bond, the *c* or *t* indicates *cis* or *trans* and the 18:1 indicates octadecenoic acid. The remaining numbers indicate position of the deuterium label and number of deuterium atoms/molecule.

were used to compare the relative use of each fatty acid to 9*c*-18:1. Selectivity values were calculated by determining the logarithm of the 12*t*-18:1/9*c*-18:1 ratio or 12*c*-18:1/9*c*-18:1 ratio found in the lipid fraction divided by the ratio in the fed mixture. Based on the standard deviation obtained for standard mixtures, the accuracy of the selectivity values is better than ±0.02. Positive selectivity values indicate a preferential incorporation of 12*t*-18:1 or 12*c*-18:1, and negative values indicate a discrimination against incorporation of 12*t*-18:1 or 12*c*-18:1 compared to 9*c*-18:1. The total amount of labeled fat incorporated into the various samples varies with time and lipid class. Unless otherwise indicated, selectivity data used in the figures and tables are for the samples that contained the maximal amount of labeled fat.

RESULTS

Plasma Lipids

Selectivity values for all plasma lipid classes are plotted in Figure 1.

A striking feature of Figure 1 is the very strong incorporation of 12*c*-18:1 into plasma PC, sphingomyelin (SM), and lysophosphatidylcholine (LPC). Positive selections for 12*c*-18:1 and 12*t*-18:1 (i.e., positive values) were also found for phosphatidylserine (PS) and phosphatidylethanolamine (PE). In contrast to these positive 12*c*-18:1 and 12*t*-18:1 selectivities, neutral lipid selectivities were negative. In all fractions, selectivity values for 12*t*-18:1 were more negative than the 12*c*-18:1 values. A second feature of Figure 1 is the large negative cholesteryl ester (CE) selectivities for 12*t*-18:1.

Evidence of acyl positional specificity for 12*c*- and 12*t*-18:1 incorporation into plasma PC is given in Table II. Previously reported 9*t*-18:1 selectivities are included in Table II for comparison (9). An extremely strong positive selection for 12*t*-18:1 incorporation into the 1-acyl PC position and a negative selection for transfer of 12*t*-18:1 to the 2-acyl PC position was observed. Comparison of the 12*t*-18:1 values to previously reported 9*t*-18:1 selectivities indicates that the same general distribution pattern is followed but the magnitude of the 12*t*-18:1 selectivity values is larger.

Five samples (3 plasma PC, one PE, and one CE), which contained 1.6-28% deuterated 12*c*-18:1, were analyzed by GC-MS for deuterated linoleate. Analyses of these samples did not detect desaturation of 12*c*-18:1 or 12*t*-18:1 to deuterated linoleate.

The fatty acid composition of TG, CE, PE, PC and SM plasma lipids from subject 2 are listed in Table III. The amount of sample left after GC-MS analysis of the plasma lipid fractions from subject 1 was sufficient for reliable GC analysis. The 0-hr plasma sample data, included to provide baseline data, are for blood samples drawn after the subject had fasted for 10 hr. The other data are for those samples within each lipid class that contained the maximal level of deuterated fat. Percentages listed as "other" include saturated and unsaturated C14, C20, C22, C24 and C26 fatty acids.

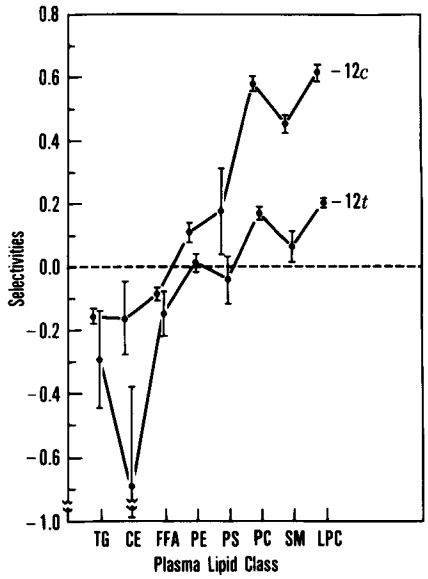

FIG. 1. Selectivity values for plasma triglyceride (TG), cholesteryl ester (CE), free fatty acid (FFA), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SM) and lysophosphatidylcholine (LPC). Fatty acid abbreviations: *cis*-12-octadecenoic acid (12*c*); *trans*-12-octadecenoic acid (12*t*). The vertical bars indicate data fluctuation due to subject variability.

TABLE II

Incorporation of 12*t*-18:1, 12*c*-18:1 and 9*t*-18:1 vs 9*c*-18:1 into Plasma 1- and 2-Acyl Phosphatidylcholine[a]

Phosphatidyl choline	Selectivity		
	12*t*/9*c*	12*c*/9*c*	9*t*/9*c*
Total	+0.170	+0.580	+0.022
1-Acyl	+0.727	+0.508	+0.562
2-Acyl	-0.900	+0.651	-0.212

[a]Average of 2 subjects.

Lipoprotein Lipids

The lipoprotein TG, CE, PC and SM samples that contained the maximal amount of deu-

TABLE III

Effect of 12*t*- and 12*c*-18:1 on Plasma Lipid Fatty Acid Composition[a]

Lipid fraction[b]	Sample time (hr)	Fatty acid composition (%)							
		16:0	16:1	18:0	*t*-18:1	*c*-18:1	18:2	20:4	Other
TG	0	31.2	2.0	13.1	2.0	11.8	24.8	9.9	5.3
	6	32.4	1.7	22.8	4.4	15.0	15.2	4.7	4.0
CE	0	12.8	3.1	1.5	0.3	15.6	53.7	5.7	7.3
	24	14.2	2.2	1.5	0.4	18.5	53.3	6.2	3.7
PE	0	21.6	8.1	14.2	0.7	9.0	6.4	20.5	19.6
	6	29.7	11.2	14.0	2.8	12.3	8.6	9.9	11.5
PC	0	32.3	2.2	16.4	2.1	10.2	22.0	10.1	4.6
	12	37.0	1.7	16.6	4.7	18.0	17.3	2.1	2.5
SM	0	38.0	5.8	9.3	0.8	8.0	16.6[c]	3.0	18.5
	12	45.6	7.7	10.0	1.0	4.1	3.4	2.5	25.6

[a]Data from subject 2.

[b]TG = triglyceride; CE = cholesteryl ester; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin.

[c]Includes 20:0 which is 3.8-4.4%.

TABLE IV

Maximal Amounts of Deuterated Fat Incorporated in Lipoprotein Lipids

Subject	Lipid[a] class	Hr	Deuterated fat content of 18:1						
			CHYLO (%)	Hr	VLDL (%)	Hr	LDL (%)	Hr	HDL (%)
1	TG	4	82.7	4	39.6	12	13.7	4	20.2
2	TG	4	83.7	4	52.9	8	32.0	6	34.1
1	CE	15	6.3	12	7.0	15	3.4	8	6.5
2	CE	15	12.3	8	36.1	24	7.2	15	9.6
1	PC	4	34.1	8	12.7	12	28.0	12	23.6
2	PC	12	30.6	8	35.2	12	46.0	12	42.7
1	SM	–	–	6	5.9	12	22.4	6	13.0
2	SM	–	–	–	–	12	15.2	15	44.8

[a]See Tables I and III for abbreviations.

terium-labeled fatty acids in the octadecenoic acid portion are listed in Table IV.

Selectivity values for CHYLO, VLDL, LDL and HDL-TG, CE, PE, PC and SM samples are plotted in the following figures. The selectivities used in these figures are from those fractions that contained the maximal total amount of deuterated fats, since data for these fractions were the most accurate.

CHYLO, VLDL, LDL, and HDL cholesteryl ester selectivities for 12*t*- and 12*c*-18:1 are plotted in Figure 2. The general pattern for selectivities for each lipoprotein fraction is similar to the pattern for plasma selectivities. However, the magnitude of the various selectivity values differ. All of the selectivity values for the CE lipoprotein samples were negative except for the VLDL 12*c*-18:1, which had a small positive value. Selectivities for the 12*t*-18:1 isomer were more negative than for the 12*c*-18:1 isomer. Within the individual lipoprotein classes, the selectivities for LDL and HDL samples were the most negative.

Selectivities for lipoprotein TG samples are shown in Figure 3. These selectivities are all negative; however, the CHYLO selectivities are essentially 0.0 for practical purposes indicating little discrimination during absorption of 12*t*- and 12*c*-18:1. Large negative selectivities were found for LDL and HDL fractions with VLDL having considerably less negative selectivities. The selectivity for the 12*c*-18:1 isomer were significantly less negative than the 12*t*-18:1 isomer in all TG samples.

PC and SM selectivities for 12*t*- and 12*c*-18:1 are plotted in Figures 4 and 5. Again, the lipoprotein selectivities followed the same general pattern as the plasma selectivities except for some differences in magnitude. The 12*t*-18:1 selectivities for PC are all positive, but

the 12*c*-18:1 selectivities were 1-2.5 times larger than the 12*t*-18:1 values.

Sphingomyelin lipoprotein selectivities in Figure 5 also showed large positive selectivities and the most variation from plasma SM selectivity values. VLDL and LDL selectivities for 12*t*-18:1 were noticeably less positive than HDL selectivities. Selectivity values for 12*c*-18:1 in all the lipoprotein-SM samples were much larger than values for the 12*t*-18:1 isomer.

Selectivities for PE lipoprotein samples are plotted in Figure 6. The PE selectivities show a large amount of fluctuation resulting from subject variation, with both positive and negative selectivities occurring in the LDL samples. The lipoprotein selectivities are relatively small, which agrees with the small selectivities found for the plasma-PE values in Figure 1. Values for only one subject are given for VLDL-PE because sufficient sample was not isolated from the other subject for GC-MS analysis.

DISCUSSION

Plasma Lipids

Triglyceride selectivities are negative for both 12*t*-18:1 and 12*c*-18:1 compared to 9*c*-18:1. These negative selectivities are partially compensated by large positive selectivities

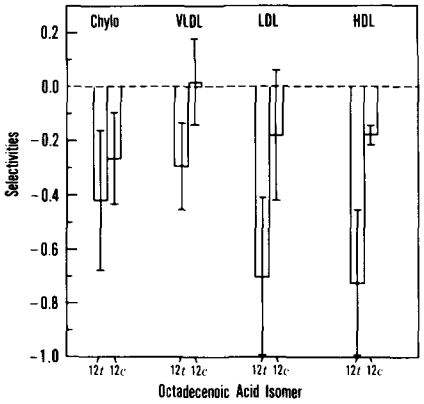

FIG. 2. Selectivity values for chylomicron (CHYLO), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesteryl esters. See Fig. 1 for other abbreviations.

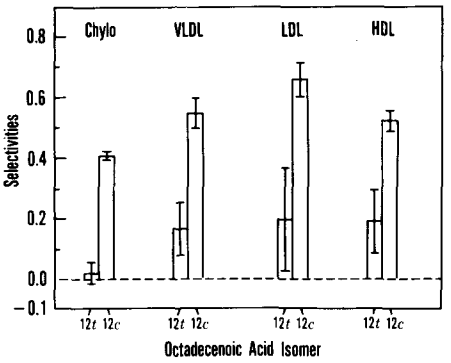

FIG. 4. Selectivity values for chylomicron (CHYLO), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) phosphatidylcholine. See Fig. 1 for other abbreviations.

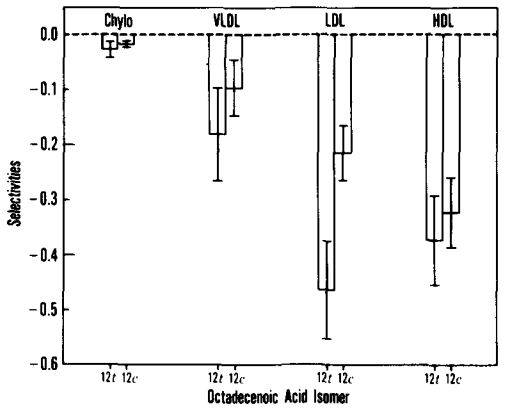

FIG. 3. Selectivity values for chylomicron (CHYLO), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) triglycerides. See Fig. 1 for other abbreviations.

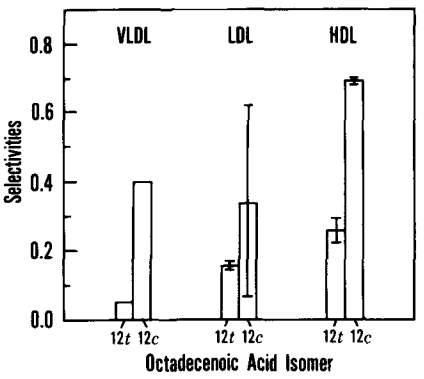

FIG. 5. Selectivity values for very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) sphingomyelin. See Fig. 1 for other abbreviations.

for 12*t*-18:1 and 12*c*-18:1 observed for most phospholipid fractions. Thus, the data in Figure 1 suggests that 12*t*- and 12*c*-18:1 may be selectively transferred from triglyceride to phospholipid. Since triglyceride selectivities for 12*t*-18:1 are more negative than those for 12*c*-18:1, the positive phospholipid selectivities for 12*t*-18:1 should be correspondingly higher if direct transfer from triglyceride to phospholipid occurs. Experimentally, positive phospholipid selectivities for 12*t*-18:1 are much less than phospholipid selectivities for 12*c*-18:1. An explanation for this difference between the 12*c*-18:1 and 12*t*-18:1 TG and PL selectivities is that 12*t*-18:1 is catabolized to CO_2 and H_2O to a greater extent than 12*c*-18:1. The negative selectivities for 12*t*-18:1 vs 12*c*-18:1 plasma free fatty acid (FFA) support the probability of a higher oxidation rate for 12*t*-18:1, since FFA are the preferred substrate for lipid oxidation in the mitochondria. A second possibility is that 12*t*-18:1 is selectively removed from the blood phospholipids by tissue lipids.

The plasma selectivities listed in Figure 1 are confirmed generally by selectivities that can be calculated from rat and chicken feeding studies (5,21,22). Considerable differences were noted in the magnitude of the selectivities from human plasma, rat liver and egg yolk samples. However, the qualitative, if not quantitative, agreement between these studies was good considering the metabolic differences between species and tissue sources.

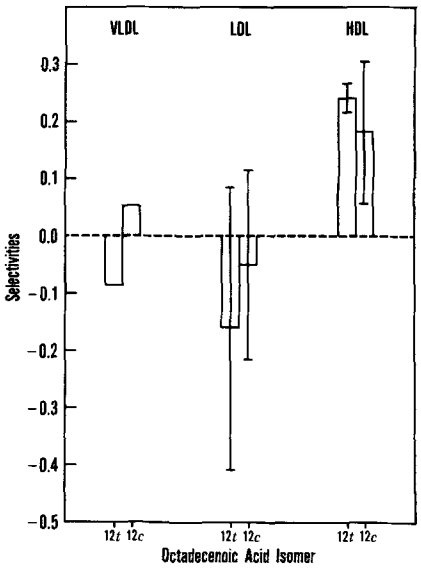

FIG. 6. Selectivity values for very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) phosphatidylethanolamine. See Fig. 1 for other abbreviations.

Positive selectivities for 12*t*-18:1 in phospholipid fractions are generally not quite as large as those reported earlier for 9*t*-18:1. Positive 12c-18:1 phospholipid selectivities are much larger than those for either 12*t*-18:1 or 9*t*-18:1. An explanation for the larger 12*c*-18:1 PC selectivity in Figure 1 is apparent from data obtained from the positional analysis of PC (see Table II). As shown in Table II, both the 12*t*-18:1 and 9*t*-18:1 isomers are selectively incorporated into the 1-acyl-phosphatidylcholine position but not into the 2-acyl position, whereas 12*c*-18:1 is selectively incorporated into both 1-acyl and 2-acyl PC positions. The difference between 12*t*/9c incorporation into the 1- and 2-acyl PC positions is 38.4-fold compared to 5.4-fold for 9*t*/9*c*. These differences in the acyl-PC selectivities for the 12*t*- and 9*t*-18:1 isomers (see Table II) indicate that both double bond configuration and double bond position are important in determining fatty acid distribution of the isomers.

The selective incorporation of 12*c*-18:1 into the 2-acyl PC position demonstrates the sensitivity of acyl CoA:PC transferase to the *cis* double bond position. Since linoleic acid (9*c*,12*c*-18:2) is reported to be incorporated predominately into the 2-acyl position of PC, 12*c*-18:1 apparently is being distributed similar to 9*c*,12c-18:2 because of the location and configuration of the 12*c* double bond. These in vivo human data are inconsistent with in vitro data reported for rat liver microsomes (23,24), but our data agree reasonably well with data from in vivo rat experiments (25). The main difference is that positive selectivities calculated from rat data are not as large as found in the human experiments. This variability in PC data suggests that considerations other than acyl CoA:PC transferase specificity are involved in fatty acid incorporation.

Selectivities calculated for plasma CE samples were negative for both 12*t*-18:1 and 12*c*-18:1. CE selectivities for 12*t*-18:1 were similar to those reported previously for 9*t*-18:1 (8). Selectivities for 12*c*-18:1 were ca. 3 times larger than either the 12*t*-18:1 or 9*t*-18:1 CE selectivities. This larger selectivity value for 12*c*-18:1 probably reflects, in part, the larger concentration of 12*c*-18:1 in the 2-acyl PC position that is available for transfer to cholesterol. The negative selectivity for 12*c*-18:1 in CE samples is surprising considering the availability of 12*c*-18:1 in the 2-acyl PC position for transfer of 12*c*-18:1 to cholesterol by cholesteryl esterase.

As noted in our previous studies with deuterated 9*t*-18:1 (8-10), deuterated 12*t*-18:1,

12*c*-18:1 and 9*c*-18:1 isomers were absent from plasma lipids 48 hr after feeding. Nonreversible accumulation of 12-18:1 positional isomers in the plasma lipid classes is not indicated by this data.

Evidence for desaturation of 12*c*-18:1 to 9*c*,12c-18:2 has been reported in pig and hen liver extracts, in algae *(Chlorella)* and in goat mammary gland extracts (26). In other species investigated, desaturase activity was much less in rabbit and mouse liver and no activity was detected in rat or hamster liver extracts or in yeast *(Candida)* (26). In our study, deuterated linoleate could not be detected in plasma samples that contained varying amounts of deuterated 12*c*-18:1 and 12*t*-18:1. The methodology used was sensitive enough to detect 0.5% deuterated linoleate in the undeuterated linoleate fraction in these samples. These data indicate either that significant 12*c*-18:1 desaturase activity is not present in human tissue or that its activity is depressed because of exogenous linoleic acid.

The effect of 12-18:1 isomers on the fatty acid composition of plasma lipids is shown in Table III. The CE samples incorporated relatively small amounts of deuterated fat compared to the other plasma lipid classes and exhibited negative selectivities for both 12*t*- and 12*c*-18:1. Thus, as may be expected, the fatty acid compositions of the 0- and 24-hr CE samples were not significantly different. In contrast, the percentage of 18:2 in TG, PC and SM were reduced considerably when 12*t*- and 12*c*-18:1 were incorporated into these plasma lipids. The percentage of 20:4 in PE and PC was also dramatically reduced. These reductions in 18:2 and 20:4 percentages were much larger than the corresponding increase in the 18:1 percentage and may partially explain the observation that hydrogenated fats seem to increase essential fatty acid requirements (27). Increases in the percentage of 16:0 also accompanied the decrease in the percentage of polyunsaturated fatty acid in TG, PC and SM samples. These results, along with the positive selectivities for 12*c*-18:1, support the concept that the 12*c*-18:1 isomers may have the capability to partially masquerade as 9*c*,12*c*-18:2.

Lipoprotein Lipids

Despite the realtively large contribution of isomeric fats to the American diet, there have been no human studies that have reported the distribution of individual isomeric fatty acids among individual lipoprotein lipid fractions. Investigations in this area would seem potentially valuable in light of the widely reported correlation between LDL and HDL cholesterol levels and atherosclerosis (28,29).

The 12*t*-18:1, 12*c*-18:1 and 9*c*-18:1 fatty acids are incorporated approximately equally into CHYLO-TG (see Fig. 3). Since 9*c*-18:1 has been shown to be ca. 97% absorbed (30), 12*t*-18:1 and 12*c*-18:1 must also be ca. 97% absorbed, because the selectivity values are close to 0.0. CHYLO-TG contain a maximum of 83.2% deuterated fatty acids compared to a maximum of 46.6% in VLDL-TG, 22.9% in LDL-TG and 27.2% in HDL-TG (see Table IV). For a meal containing 20 g of triglycerides, approximately equal amounts of triglycerides were reported to be incorporated into CHYLO and VLDL during absorption (31). Our results show that a maximal total deuterated fatty acid incorporation occurs in the 4-hr CHYLO-TG and 4-hr VLDL-TG samples. The concept that both CHYLO and VLDL are being formed in the intestinal mucosal cells is supported by the data in Table IV. Maximal incorporation of deuterated fats into HDL-TG was found in the 4-hr (subject 1) and 6-hr (subject 2) samples, which indicates HDL also may be formed in appreciable amounts in the intestinal mucosal cell.

The short half-life of ca. 15 min reported for CHYLO (32) could also account for the total deuterated TG in VLDL. If CHYLO-TG and VLDL-TG are formed at the same time by the intestine in about equal amounts, it is surprising that selectivities for each fraction are not nearly identical. Based on the lower VLDL-TG selectivities, CHYLO-TG apparently is hydrolyzed to FFA and then incorporated into both VLDL-TG and phospholipids after the CHYLO-TG has entered the blood stream.

The selectivity values shown in Figures 2-6 for liproprotein lipid fractions confirm the selectivity values found in total plasma samples, although variations from the plasma values were found. Differences between the VLDL, LDL and HDL selectivities for individual lipid classes provide insight into the association of various lipid fractions with individual lipoproteins.

Incorporation of 12*t*-, 12*c*- and 9*c*-18:1 into individual lipid fractions was dependent on the lipoprotein fraction to which the lipid was associated (see Fig. 2-6). Since LDL and HDL half-lives are long compared to deuterated fatty acid life times, a rapid exchange or turnover of associated lipids must occur as previously suggested (33). However, there were significant differences in selectivities between all but 4 of the lipoprotein samples plotted in Figures 2-6, which indicates that exchange is not random since the deuterated lipids are not equally distributed among CHYLO, VLDL, LDL and

HDL. This lack of equilibration indicates that individual fatty acids are either directed into individual lipid classes associated with specific lipoproteins or that lipid fractions containing specific fatty acids are exchanged preferentially between lipoprotein fractions. In either case, the mechanism that controls this distribution must be sensitive to properties associated with the fatty acid double bond position or configuration. The reason for the variation in selectivity for a specific lipid fraction associated with different lipoproteins is unknown. The fact that selection obviously occurs supports the concept that the various physical and chemical properties of specific fatty acids have an important biological role.

ACKNOWLEDGMENTS

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METHODS

Quantitative Determination of Isomeric Glycerides, Free Fatty Acids and Triglycerides by Thin Layer Chromatography-Flame Ionization Detector System[1]

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ABSTRACT

Partial glyceride mixtures, which include 1-monoglyceride, 2-monoglyceride, free fatty acid, 1,2-diglyceride, 1,3-diglyceride and triglyceride, could be separated from each other on a 3% boric-acid-impregnated Chromarod S-II (silica gel sintered quartz rod) with either chloroform/acetone (96:4, v/v) or chloroform/acetone/acetic acid (100:1:1, v/v) as the developing solvent mixtures. The components separated on the boric-acid-impregnated rod were automatically quantitated in a hydrogen flame ionization detector (Iatroscan). The relative responses of 1,2-diglyceride, 1,3-diglyceride, free fatty acid and triglyceride were slightly lower than theoretical responses based on weight percentage, whereas 1-monoglyceride and 2-monoglyceride showed slightly higher responses. These responses were converged within a maximal error of 5-10% (SD). Boric-acid-impregnated rods could be used repeatedly, ca. 5 times without any reconditioning procedure.

ABBREVIATIONS

Monoglyceride, MG; diglyceride, DG; free fatty acid, FFA; triglyceride, TG; monostearin, MS; monopalmitin, MP; distearin, DS; stearic acid, SA; tripalmitin, TP; chloroform, C; acetone, A; acetic acid, HAc.

INTRODUCTION

Several methods have been proposed for the quantification of the components separated on thin layer chromatograms using the hydrogen flame ionization detector (FID) (1-4).

Okumura et al. (5) developed an adsorbent sintered thin layer chromatographic quartz rod (0.9 × 150 mm) which consists of silica gel powder fused by fine glass powder as the binding agent. These rods are now commercially available under the trade name Chromarod (Iatron Lab. Inc., Tokyo, Japan). In addition, an automatic scanner which contains a hydrogen-FID for sample detection also is commercially available under the trade name Iatroscan from Iatron Lab. Inc.

We have initiated the application of the Chromarod-Iatroscan system for the quantitative analyses of a variety of lipids (6-8). Recently, other investigators also have described the application of this system for lipid research (9-14).

Adsorbent sintered rod has many advantages compared to adsorbent coated rods. Generally, sintered rods are reusable without any of the reconditioning procedures. In addition, since the sintered rod is sturdy, the properties of the adsorbent could be modified by immersing in a liquid reagent solution. In this manner, reagent-impregnated rods can be prepared routinely.

This paper describes a method for the quantitative estimation of the composition of partial glyceride mixture including 1-monoglyceride, 2-monoglyceride, 1,2-diglyceride, 1,3-diglyceride, free fatty acid and triglyceride using the boric-acid-impregnated Chromarod-Iatroscan system.

EXPERIMENTAL PROCEDURES

Apparatus and Operating Conditions

The equipment used was the Iatroscan TFG-10 (initially named Thinchrograph). The

[1]Part of this investigation was reported at the 2nd JOCS-AOCS joint meeting, San Francisco, May 1979.

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differential and integral curves were recorded by a Hitachi 056 2-pen recorder (Hitachi Ltd., Tokyo, Japan).

Automatic scanning of Chromarod by Iatroscan was performed under the following conditions: flow rate of hydrogen, 125 ml/min; flow rate of air, 2000 ml/min; chart-drive speed, 240 mm/min; voltage of detector, 50 mV; and voltage of recorder, 100 mV.

Rods

Chromarod S-II was used which carries a thin adsorbent (5 μ in diameter, silica gel powder) layer fused with fine glass powder as a binding agent on a 0.9 mm × 150 mm quartz rod. Boric-acid-impregnated rods were prepared by immersing the Chromarod S-II in 3% boric acid solution. After the rods were used 5 or more times, they were immersed in a chromic-sulfuric acid solution overnight and then washed under running water. The washed rods can be reused.

Materials

1-Monostearin and tripalmitin were purchased from Nihon Glyceride Kogyo Co., Ltd. (Tokyo). 2-Monopalmitin was obtained from Serdary Research Lab. (Ontario), and 1,2-distearin and 1,3-distearin from Gasukuro Kogyo Co., Ltd. (Tokyo). Stearic acid was supplied by Nippon Oil and Fat Co., Ltd. (Tokyo). Each lipid was homogeneous on thin layer chromatograms. The following standard mixtures were used for evaluation purposes: standard mixture I contained 1-monostearin/2-monopalmitin/stearic acid/1,2-distearin/1,3-distearin/tripalmitin of weight ratio 14.7:12.4:17.2:13.9:13.3:28.5, respectively. The weight percentages of 1-monostearin and 2-monopalmitin in standard mixture II were 54.2 and 45.8, respectively.

Lipase hydrolysates of olive oil were prepared by the following process. One g of olive oil (Kanto Kagaku Co., Ltd., Tokyo) was incubated with 6 mg of MY lipase (gift from Meito Sangyo Co., Ltd.) in 25 ml of water at 37 C. Reaction mixtures after lipolysis at 0 time and 1, 2, 3, 4 and 7 hr, respectively, were partitioned with chloroform/methanol (2:1).

All organic solvents were distilled before use. Other organic and inorganic reagents were of analytical grade or of the highest quality commercially available.

Methods

Boric-acid-impregnated rods were activated by passing through the automatic FID scanner (Iatroscan) just prior to use. They were then spotted at the origin with ca. 1 μl of the sample solution containing 20-30 μg of lipids. The rods were developed with a solvent mixture until the solvent front travelled to 10 cm from the origin. After developing, the rods were dried on phosphorus pentoxide (P_2O_5) in vaccuo for 5 min and then scanned by Iatroscan. The developing solvents were (a) chloroform/acetone (96:4, v/v); (b) chloroform/acetone/acetic acid (100:1:1, v/v).

RESULTS AND DISCUSSION

In order to quantify the amounts of 1-monoglyceride and 2-monoglyceride, the boric-acid-impregnated silica gel TL Chromatographic technique (15) was used. In addition to the complete separation of the isomeric glycerides, stearic acid and tripalmitin were also resolved from each other on a 3% boric-acid-impregnated Chromarod S-II using solvent mixture a or b. A typical chromatogram is shown in Figure 1. Stearic acid migrated between 2-monopalmitin and 1,2-distearin with solvent mixture a. It migrated between 1,2-distearin and 1,3-distearin when the rod was developed with solvent mixture b. The difference in migration rate was probably caused by the small amount of acetic acid present in solvent mixture b.

We initially were concerned with the isomerization of monoglycerides and diglycerides during chromatography. However, when pure 1-monopalmitin and 2-monopalmitin were chromatographed on boric-acid-impregnated

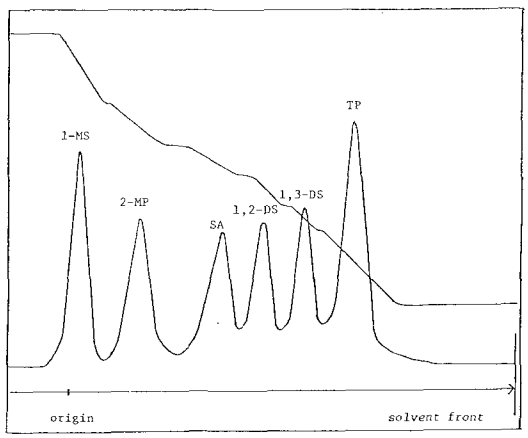

FIG. 1. Chromatogram of a partial glyceride mixture on boric-acid-impregnated Chromarod S-II. Boric acid impregnated Chromarod S-II was prepared as described in Experimental. See Abbreviations. Sample: standard mixture I. Mobile phase: solvent mixture a.

TABLE I

Comparison between Percentages of Peak Area Obtained from Boric Acid Impregnated Chromarod S-II-Iatroscan System and that of Weight for Partial Glycerides and Fatty Acid

Lipids	Percentage					
	1-MS	2-MP	1,2-DS	1,3-DS	SA	TP
Average of peak area percentage	19.0	15.9	12.1	12.5	14.3	26.2
± sd	1.2	0.6	1.3	0.5	0.5	2.2
Weight percentage	14.7	12.4	13.9	13.3	17.2	28.5

The figures of mean value and sd were obtained from 13 rods. See Abbreviations. Sample: standard mixture I.

silica gel plates (Replate) (16), a single characteristic spot was always obtained for each component. Similar results were obtained for diglycerides. These observations indicated that isomerization of mono- and diglycerides did not occur during the chromatographic process.

Comparison between the percentage of relative peak area and weight for 1-monostearin, 2-monopalmitin, 1,2-distearin, 1,3-distearin, stearic acid and tripalmitin on the boric-acid-impregnated Chromarod S-II-Iatroscan system are shown in Table I. Relative percentages of peak area for 1,2-distearin, 1,3-distearin, stearic acid and tripalmitin were slightly lower than that of weight, whereas in the monoglycerides (1-monostearin, 2-monopalmitin), slightly higher peak area percentages were seen. Table I also shows that the reproducibility of peak area percentages was within 5-10% (SD).

The influence of repeated usage of the boric-acid-impregnated Chromarod S-II was examined with standard mixture II (Table II). Five rods were spotted and developed at once. Even after using these rods 5 times, the chromatographic resolution, relative area percentages of 2 components and standard deviations were almost the same. Therefore, the boric-acid-impregnated rods could be used at least 5 times without any reconditioning procedure.

We proposed an application of the boric-acid-impregnated silica gel rod TLC-FID-system for monitoring the lipase kinetics. Figure 2 shows a typical chromatogram of lipase hydrolysate of olive oil. An aliquot (1 μl, 20-30 μg of lipids) of chloroform/methanol extract from enzyme reaction mixture was used for this chromatogram. After a 3 hr treatment lipase with olive oil, 60% of triglycerides was degraded and free fatty acids were liberated. Even the presence of a large amount of free fatty acids in the sample, 1-monoglycerides, 2-monoglycerides, 1,2-diglycerides and 1,3-diglycerides were clearly separated. Figure 3 shows the course of lipase hydrolysis of olive oil. The

TABLE II

Reproducibility of Percentages of 1-Monostearin and 2-Monopalmitin on Repeatedly Used Boric Acid Impregnated Chromarod S-II

Run no.	Peak area (%)	
	1-Monostearin	2-Monopalmitin
1	56.9 ± 2.2	43.1 ± 2.2
2	55.8 ± 2.1	44.2 ± 2.1
3	55.6 ± 1.8	44.4 ± 1.8
4	56.1 ± 1.2	43.9 ± 1.2
5	55.9 ± 1.0	44.1 ± 1.0
6	57.0 ± 1.8	43.0 ± 1.8
7	56.8 ± 1.5	43.2 ± 1.5
8	57.3 ± 1.1	42.7 ± 1.1
9	58.0 ± 1.9	42.0 ± 1.9
10	58.9 ± 2.6	41.1 ± 2.6
Wt %	54.2	45.8

The figures of mean value ± sd were obtained from 5 rods. These rods were repeatedly used 10 times without employing the reconditioning procedure. Sample: standard mixture II.

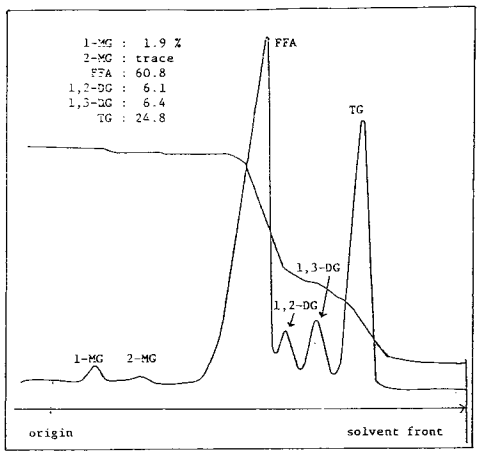

FIG. 2. Chromatogram of lipase hydrolysate of olive oil on boric-acid-impregnated Chromarod S-II. Hydrolyses procedure was employed as described in Experimental. The sample for chromatogram illustrated in the figure was obtained from the reaction mixture after 3 hr. The rod was prepared as described in Experimental. Mobile phase: solvent mixture a. See Abbreviations.

quantitative profile of enzymatic degradation products at each sampling time could be rapidly (within 30 min) and sensitively (20-30 μg of lipids) assayed using this system.

In summary, we have established a sensitive and reliable method for the determination of partial glyceride mixtures. This method should be useful in studying the composition of glycerides in a number of natural products, and in studying the kinetics of lipase actions on natural oils.

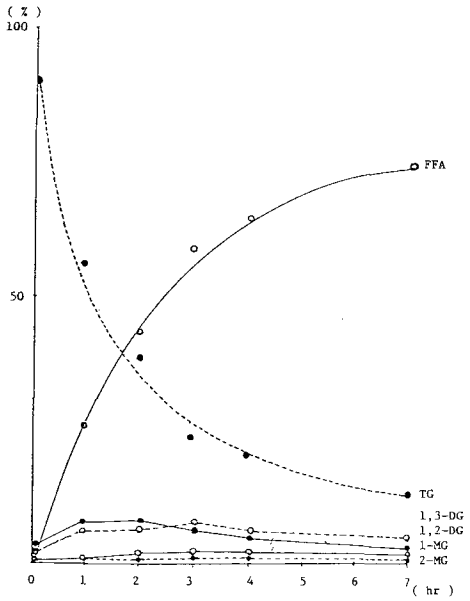

FIG. 3. The tracing of hydrolytic process by boric-acid-impregnated Chromarod S-II-latroscan system. The hydrolysis products were obtained by incubation described in Experimental. See Abbreviations.

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COMMUNICATIONS

Occurrence of Unusual Hexadecenoate Fatty Acids in Hepatoma Lipids

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ABSTRACT

Cis-hexadecenoates isolated from rat liver and hepatoma 7288CTC lipids were analyzed for positional isomers by ozonolysis and capillary gas liquid chromatography. In addition to the Δ6, Δ7, Δ9 and Δ11 isomers found in both tissues, the hepatoma neutral and polar lipids contained relatively high percentages of Δ12 and Δ14 hexadecenoates that were virtually absent from liver. The occurrence of these unusual fatty acids may result from an error in lipid metabolism in the hepatoma.

INTRODUCTION

Except in triglycerides (1-3), the hexadecenoate fractions of liver and hepatoma lipids represent a small percentage of the total, which is one of the reasons why this monoene fraction has not been examined in detail. We became interested in the positional isomers of the hexadecenoate fraction when studies with dietary octadecenoates suggested the presence of a chain-shortening process to yield hexadecenoates (4). More recently, we encountered what apparently was some unusual hexadecenoate isomers in the hepatoma lipids isolated from host animals fed a low level of methyl 2-hexadecynoate (5).

In this study, we have examined the hexadecenoate fractions from liver and hepatoma for positional isomers by 2 methods. The data indicate that the hepatoma contains some unusual hexadecenoate isomers.

MATERIALS AND METHODS

Normal rat livers and 7288CTC hepatomas were obtained from groups of animals that had been maintained on a commercial chow diet (6), a fat-free diet supplemented with 0.5% safflower oil (6), or a fat-free diet supplemented with 0.5% safflower oil and 0.1% methyl 2-hexadecynoate (5) for 4 weeks. Lipids were extracted by the Bligh and Dyer procedure (7), separated into neutral lipid and phospholipid fractions by silicic acid chromatography (8), individual lipid classes isolated by thin layer chromatography (TLC), methyl esters prepared and analyzed by gas liquid chromatography (GLC) as described previously (9). The *cis* monoene fraction was resolved by silver nitrate TLC and the hexadecenoate fraction was isolated by preparative GLC on a nonpolar column (10). The position of the double bond was determined by GLC analysis of the ozonide cleavage products described previously (11). Ozonides were prepared by a modification (11) of the Beroza and Bierl procedure (12). The hexadecenoate fractions were also analyzed by capillary GLC using a 200-ft × 0.01-in. (id) stainless column coated with a 10% solution of diethylene glycol succinate in chloroform. The analyses were carried out at 170 C in a Varian 3700 chromatograph designed for capillary column operation.

RESULTS AND DISCUSSION

Generally, ozonolysis is a satisfactory method for the quantitative analysis of double bond position in most monoenes. However, when the double bond is at the Δ7 position or nearer to the carboxyl group, the yield of the aldehyde-ester fragment is very poor and the aldehyde fragment must be used to estimate the percentage of these isomers (1). In addition, the 14-carbon aldehyde-ester, arising from a Δ14 hexadecenoate, has the same approximate retention time as any unreacted hexadecenoate making identification of this isomer hard to confirm. Because some of our samples apparently contained isomers in these 2 general positions, we also analyzed the hexadecenoates intact by capillary GLC for positional isomers (Fig. 1). The *cis*-hexadecenoates from normal liver lipids could be resolved into 3 peaks (Fig. 1A) whereas the hexadecenoates from the hepatoma lipids gave rise to at least 4 discrete peaks (Fig. 1B). Analyses of the first half (Fig. 1C) and the last half (Fig. 1D) of the eluting hexadecenoate sample (from Fig. 1B) collected

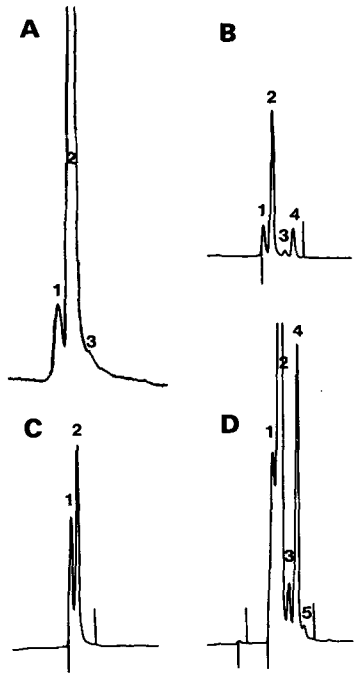

FIG. 1. Representative gas liquid chromatographs of hexadecenoate positional isomers isolated from normal liver neutral lipids of Chow-fed animals (A), hepatoma neutral lipids from Chow-fed host animals (B), first half of the peak of the hepatoma neutral lipid sample (B) collected from a preparative chromatograph (C), and the last half of the collected peak (D). Numbered peaks: 1 = Δ6 and Δ7 hexadecenoates; 2 = Δ9 hexadecenoate or palmitoleate; 3 = Δ11 hexadecenoate 4 = Δ12 and Δ14 hexadecenoates; and 5 = tentatively identified as Δ15 hexadecenoate.

from a preparative chromatograph showed isomeric enrichment in the 2 fractions. Ozonolysis data (Table I) in conjunction with the GLC data indicated that peak 1 consisted of the Δ6 and Δ7 isomers, peaks 2 and 3 were composed of the Δ9 and Δ11 isomers, respectively, and peak 4 represented the Δ12 and Δ14 isomers. Traces of a Δ15 isomer may have given rise to peak 5 in Figure 1D, but this was not confirmed by ozonolysis. Our assigned order of the hexadecenoate positional isomer elution is in agreement with the elution order of octadecenoate positional isomers on polyester capillary columns reported by Ackman and Hooper (13-14). Generally, the isomeric percentages from ozonolysis and the GLC (Table I) agreed reasonably well. Closer agreement between ozonolysis and GLC values can be calculated by taking into account a greater detector response of the longer chain aldehyde-esters because of the larger amount of carbon per molecule. Such calculated values would probably still remain in error since purified aldehyde-ester standards are not available for accurate detector calibration. Despite the lack of precise quantitative values, we feel the data are reliable.

The percentages of the hexadecenoates in the total fatty acids of the various fractions are given in parentheses in Table I. Neutral lipids from animals fed chow and the low fat diets contained much higher levels of hexadecenoates than the hepatoma. Unlike liver, the hepatoma is generally unresponsive to diet (1), but a test diet containing methyl 2-hexadecynoate produced a 2-fold increase in hepatoma hexadecenoates (5). The increased level of hepatoma hexadecenoates was minimal compared to the massive accumulation of hexadecenoates in liver, but the elevated levels had little effect on the positional isomer distribution in either tissue (5).

Analysis of hexadecenoates from liver neutral lipids and phospholipids indicates that palmitoleic, the Δ9 isomer, represented 85-90% of the total with the Δ6, Δ7 and Δ11 isomers making up most of the balance (Table I). When large hexadecenoate fractions from liver phospholipids were analyzed, some small peaks corresponding to C_{12} and C_{14} aldehyde-esters were detected. We have analyzed the hexadecenoates from triglycerides, phosphatidylcholines and phosphatidylethanolamines of a variety of tissues from animals maintained on chow and low-fat diets, but the Δ12 and Δ14 isomers were seldom observed and then only in trace amounts, which were not reported (1-3,5). Spence (15,16) has also examined the hexadecenoates from a number of rat tissues, but the presence of Δ12 and Δ14 isomers was not reported. All these data strongly suggest that the Δ12 and Δ14 hexadecenoate isomers are present in very low amounts in the lipids of normal rat tissue.

In contrast to liver, hepatoma neutral lipids and phospholipids from host animals fed 2 different diets contained relatively high percentages of the Δ12 and Δ14 isomers, in addition to the Δ6, Δ7, Δ9 and Δ11 hexadecenoate isomers (Table I). The presence of the high delta (positional isomers with the double bond greater than 11 carbon atoms from the carboxyl group) hexadecenoates in the hepatoma lipids was confirmed by the capillary GLC data (Table I and Fig. 1). The absence of the Δ12 and Δ14 isomers in the first half of the hexadecenoate peak collected from the preparative chromatograph by both methods of analysis eliminates the possibility of their being artifacts. A number of other procedures, e.g., argentation TLC, GLC on polar and nonpolar

TABLE I

Percentage Distribution of *Cis*-Hexadecenoate Positional Isomers Present in Hepatoma and Liver Lipids

Source and diet	Method of analysis	Percentage of positional isomers[a]					
		Δ6	Δ7	Δ9	Δ11	Δ12	Δ14
Liver, total NL, chow[b] (9.2 ± 1.3)[c]	OZO	2.0 ± 0.4	6.0 ± 2.4	92.1 ± 2.7			
		8.1 ± 1.8					
	GLC			89.1 ± 2.4	2.8 ± 0.7		
Liver, total PL, chow (2.8 ± 0.21)	OZO	2.2 ± 0.5	3.9 ± 0.3	87.1 ± 2.2	2.6 ± 0.1	1.3 ± 0.0	3.0 ± 0.8
		9.0 ± 1.1				1.2 ± 0.2	
	GLC			85.4 ± 0.9	4.4 ± 0.5		
Liver, total NL, low fat (13.0)[d]	OZO	5.0	3.8	89.8	1.3		
		7.6					
	GLC			88.9	3.5		
Liver, total PL, low fat (3.5)	OZO	3.7	2.3	91.1	1.4	0.7	0.7
		1.6					
	GLC			95.1	3.3		
Hepatoma, total PL, chow (1.8 ± 0.2)	OZO	2.2 ± 0.1	8.7 ± 1.3	59.2 ± 4.1	3.4 ± 0.8	15.5 ± 3.0	11.0 ± 2.8
		14.6 ± 2.0				11.7 ± 3.0	
	GLC			69.5 ± 3.0	4.2 ± 0.6		
Hepatoma, total NL, chow (1.5)	OZO	1.3	8.6	54.2	5.2	19.2	3.0
		14.9				13.1	
	GLC			70.9	1.1		
Hepatoma, total NL, chow 1st half of 16:1 peak	OZO	2.9	50.4	46.6			
		39.8					
	GLC			60.2			
Hepatoma, total NL, chow Last half of 16:1 peak	OZO	0.8	12.0	53.0	3.9	23.0	7.3
		15.6				19.2	
	GLC			61.1	3.8		
Hepatoma, total PL, test[e] (2.2)	OZO	3.2	10.6	69.0	2.0	10.8	4.4
		7.3				7.0	
	GLC			82.9	2.7		
Hepatoma, total NL, test (3.0)	OZO	3.5	2.8	76.9	1.1	8.6	6.8
		8.2				6.2	
	GLC			84.3	1.4		
Hepatoma, total NL, test 1st half of 16:1 peak	OZO	5.3	6.1	88.6			
		14.0					
	GLC			86.0			
Hepatoma, total NL, test Last half of 16:1 peak	OZO	3.6	4.9	51.9	3.3	28.2	8.1
		6.3				22.8	
	GLC			67.8	3.2		

Abbreviations: NL = total neutral lipids; PL = total phospholipids; PC = phosphatidylcholines; GLC = gas-liquid chromatography; 16:1 = methyl hexadecenoate, OZO = ozonolysis.

[a]Means with standard deviations are from the individual analysis of 3 or more animals whereas means without standard deviations represent analysis of a pooled sample obtained from 3 or more animals.

[b]The chow diet was Wayne Lab-Blox.

[c]The percentage of the hexadecenoates of the total methyl esters is given in parentheses.

[d]Composed of a fat-free diet supplemented with 0.5% safflower oil.

[e]The test diet consisted of the fat-free diet supplemented with 0.5% safflower oil and 0.1% methyl 2-hexadecynoate.

columns, hydrogenation, support the conclusion that hexadecenoates from hepatoma 7288CTC lipids contain a high percentage of the Δ12 isomer, and to a lesser degree the Δ14 isomer, relative to normal liver.

The origin of the hexadecenoate isomers is of considerable interest and may represent a more important question than the effect these unusual fatty acids might have on a biological system. Dietary origin of the unusual isomers can be discounted. The chow diet and the lipids added to the fat-free diet have been shown to contain primarily palmitoleate with the Δ7 and Δ11 isomers making up the balance (2). The endogenous origin of most hexadecenoate isomers is known. Palmitoleate, the major isomer, arises from the microsomal aerobic desaturation of the corresponding saturated fatty acid, palmitate (17,18). The Δ11 isomer is probably derived from the elongation of myristoleate, similar to the origin of vaccenate from palmitoleate (18). Biosynthesis of both the Δ9 and Δ11 hexadecenoates has been shown to occur in hepatoma 7288C (19), the origin of the host-grown hepatoma (7288CTC) used in these studies. The Δ7 isomer probably arises from oleate by the recently observed chain-shortening process (4). The origin of the Δ6 isomer is unknown, but we have speculated on several possibilities, of which Δ6 desaturation of palmitate appeared the more likely (3).

Since the occurrence of the Δ12 and Δ14 hexadecenoates has not been established previously, their origins are unknown but their relatively high levels in the hepatoma lipids may result from an error in metabolism of this neoplasm. Only a few microorganisms of the *Bacillus* species (20) are known to produce unsaturated fatty acids with a double bond located at an even carbon-number position, and then only up to the Δ10 position. This would appear to rule against the hepatoma having acquired the ability to desaturate palmitate directly at the Δ12 or Δ14 positions. The more attractive concept is one involving meatbolic error early in fatty acid biosynthesis because the unsaturation occurs at an even carbon-number position. The Δ14 and Δ12 isomers would be produced if 2,3 unsaturated acyl-ACP was not reduced after the first and second malonyl-CoA condensation, respectively. Formation of the *cis*-Δ2 isomer instead of the normal *trans*-unsaturated acyl-ACP might contribute to bypassing enoyl-ACP reductase. The *cis*-Δ2 isomer could result from the loss in stereospecificity of 3-hydroxyacyl-ACP hydratase. If multiple forms of this enzyme exist in the hepatoma as in *Escherichia coli* (18), a loss of specificity in the 3-hydroxybutyryl-ACP hydratase isozyme, which acts only on short chains, would agree with the observed absence of the unsaturation at the Δ8 and Δ10 positions of the hexadecenoates. It is possible that a loss in the stereospecificity of 3-ketoacyl-ACP reductase could result in the formation of the L-3-hydroxyacyl isomer in addition to the normally produced D-isomer. Although there may be other explanations, the one described apparently is a logical place to look for an error in metabolism that could account for the occurrence of the unusual hexadecenoate isomers found in the hepatoma lipids.

ACKNOWLEDGMENTS

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Cutin Acids: Synthesis and Mass Spectrometry of Methyl 16-Hydroxy-7-oxo-, 16-Hydroxy-8-oxo-, 16-Hydroxy-9-oxo-, 16-Hydroxy-10-oxo- and 7,16-, 8,16-, 9,16- and 10,16-Dihydroxyhexadecanoates[1]

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ABSTRACT

Methyl esters of 8 16-carbon acids which occur in cutin of plants have been synthesized from methyl 16,17-dihydroxy-7-oxo- and 16,17-dihydroxy-9-oxoheptadecanoates. 16-Hydroxy-7-oxo- and 16-hydroxy-9-oxohexadecanoates were prepared by lead tetraacetate cleavage followed by sodium triacetoxyborohydride reduction; 16-hydroxy-8-oxo- and 16-hydroxy-10-oxohexadecanoates were prepared by successive ketalization, ester reduction, diol cleavage, acylation, oxidation and methanolysis. Dihydroxy esters were prepared by reduction of oxo esters with sodium borohydride. Mass spectra of TMS ethers of the esters, of pyrrolides and of TMS esters of pyrrolidides have been compared. Mass spectra of mixtures of hydroxyoxo derivatives and of dihydroxy derivatives show that correction factors are required for quantitative analysis, particularly when 7-substituted isomers are present.

INTRODUCTION

The surface of plants is protected by a layer called the cuticle; the outer part consists of the epicuticular wax and the inner part consists of the cuticular membrane composed of cutin. The principal functions of the cuticle are to separate the plant from the environment and to maintain rigidity, thus holding the plant together (1).

The cutin of the cuticular membrane has been repeatedly investigated and analyzed. It is thought that, in many plants, it is principally composed of polyesters derived from hydroxy fatty acids, containing one or more hydroxyl groups, but ether and peroxide linkages also may be present. Alkaline hydrolysis of cutin yields complex mixtures of oxygenated acids and, depending on the species of plant, these may be mainly C_{16} or C_{18} acids (2).

The first C_{16} hydroxy acid isolated and identified was 10,16-dihydroxyhexadecanoic acid from cutin of an *Agave* species (3); later the same acid was obtained from olive leaves (4).

Subsequently, however, investigations using gas chromatography-mass spectrometry (GC-MS) showed that the dihydroxy C_{16} cutin acids were mixtures of 7,16-, 8,16-, 9,16- and 10,16-dihydroxy isomers (5-7). Hydroxyoxo C_{16} acids, 16-hydroxy-7-oxo, 16-hydroxy-8-oxo, 16-hydroxy-9-oxo and 16-hydroxy-10-oxo isomers also have been found in some cutins, particularly in that of lemons (8).

These cutin acids apparently had never been synthesized and the GC-MS studies showed that pure individual isomers had probably never been isolated (5). In estimating the proportions of isomers in natural mixtures by MS, it had to be assumed that intensities of fragments were proportional to amounts of isomers (5). For these reasons, and because convenient synthetic routes to the 4 dihydroxy and the 4 hydroxy-oxo hexadecanoic acids already mentioned were apparently available, syntheses of methyl esters of the 8 acids have been carried out. Preparations and MS properties of the products are reported here.

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RESULTS AND DISCUSSION

Synthesis

To facilitate the synthetic work, the 4 hydroxyoxo esters were to be prepared first and reduced to the corresponding dihydroxy esters with sodium borohydride. As a further simplification, syntheses were arranged so that just 2 key intermediates and 2 routes (A and B, Figs. 1 and 2) applied to each would yield the 4 isomers.

The intermediates were methyl 16,17-dihydroxy-7-oxo- and 16,17-dihydroxy-9-oxo-heptadecanoates, **1** and **2**. The former was readily obtained by hydroxylation of 7-oxo-16-hexadecenoic acid, prepared from 10-undecenoyl chloride by enamine synthesis (9,10) using 1-morpholinocyclohexene; a longer reaction sequence was required for the latter. Two malonate chain extensions from 4-pentene-1-ol gave 6-heptenylmalonic acid which, when allowed to react with the acid chloride of

methyl hydrogen azelate, under the conditions of Bowman's ketone synthesis (11,12) gave 9-oxo-16-heptadecenoic acid. Dihydroxyoxo ester **2** was obtained by hydroxylation.

In synthetic route A from **1** or **2** (Fig. 1) the ester group is retained in the product. This route makes use of the bulky reducing agent sodium triacetoxyborohydride which selectively reduces the less hindered aldehyde group in the presence of a carbonyl group (13). Cleavage of the diol in **1** or **2** with lead tetraacetate gave intermediate oxoaldehydes and reduction with sodium triacetoxyborohydride gave hydroxyoxo esters **3** or **4**. Dihydroxy esters **5** or **6** were obtained by borohydride reduction. Yields from **1** or **2** were ca. 70%.

In route B (Fig. 2), the ester group of **1** or **2** becomes the terminal carbon with the primary hydroxyl group and C-16 becomes C-1 of the product. Attempts to protect the carbonyl group as the ketal (14) led to mixtures of ketone and ketal. Even when the terminal glycol was acylated and when the ketal was prepared by exchange with butanone ketal (15), only partial ketalization occurred. Reaction was carried out, however, using the mixture of intermediates. Reduction of the ester with lithium aluminum hydride, followed by cleavage of the diol, acetylation of the hydroxyl group, oxidation with chromic acid and treatment with methanolic HCl gave a mixture of hydroxyoxo ester **7** or **8** and dihydroxy ester **9** or **10** in a combined yield of 44%. The 2 types of esters were readily separated by column chromatography.

Dihydroxy ester **9** also was prepared without employing the ketal intermediate to investigate conversion of **9** to **7**, which route B gave in only low yield, by selective oxidation. Bromine had been reported to oxidize secondary alcohols preferentially to ketones (16) and application of this method to **9** gave **7** in 46% yield. Thus, the isomeric hydroxyoxo and dihydroxy esters have been synthesized in moderate-to-good yield from fairly readily available starting materials. The structures of the products followed from the synthetic routes employed but they also were confirmed by the MS study which follows.

None of the compounds had been prepared previously and only one, methyl 10,16-dihydroxyhexadecanoate, had been reported as a pure compound obtained from cutin (3,4). It is unclear whether this particular ester from *Agave* cutin has ever been examined by MS but possibly a pure isomer resulted from crystallization. The mp of the ester from *Agave* was 67.5-68.3 C (3) and 68-69 C for the sample from olive leaves (4). Ester with mp 67-68 C also has been isolated from the soft resin of shellac (17). Methyl 10,16-dihydroxyhexadecanoate prepared here had mp 63-64 C. The natural ester would be expected to be an optical isomer with probably a different mp and, in fact, the dihydroxyhexadecanoate fraction from tomato cutin, containing 79% of the 10,16-isomer, has a small positive rotation (18).

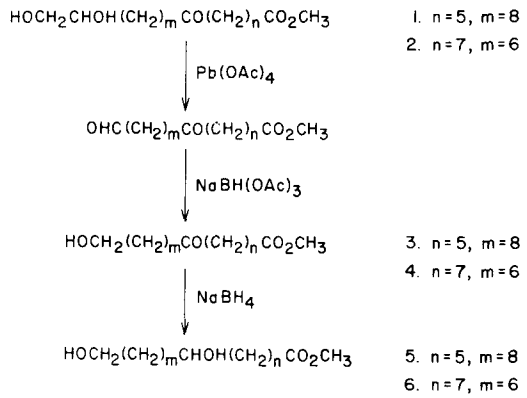

FIG. 1. Syntheses of methyl hydroxyoxo- and dihydroxyhexadecanoates by route A.

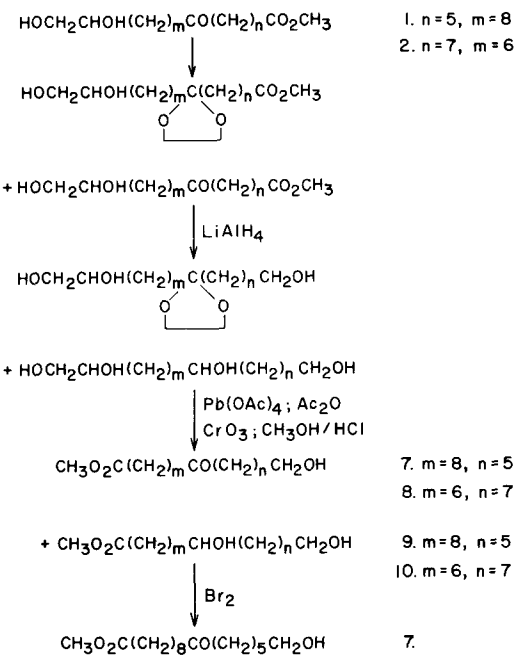

FIG. 2. Syntheses of methyl hydroxyoxo- and dihydroxy-hexadecanoates by route B.

MS and Other Properties of Hydroxyoxo and Dihydroxy Esters

During isolation of oxygenated esters from hydrolyzed cutin, fractions containing hydroxyoxo esters (8) and, more frequently, dihydroxy esters (5,19,20) were separated by thin layer chromatography (GLC) but individual isomeric components apparently were never obtained. Examination of the synthetic isomers showed that, comparing isomers with substituents on neighboring carbons, the R_f was lower the closer the substituent was to the hydroxyl end of the chain. The difference was greatest for the 9,16- and 10,16-dihydroxy isomers and these could be separated by analytical TLC. This result suggests that dihydroxy esters containing major proportions of 10,16-isomer, such as those from tomato (5) or *Vicia faba* (21), could be fractionated by TLC yielding pure 10,16-isomer.

Previous MS investigations of C_{16} cutin esters, usually as TMS derivatives, were limited to the 10,16-dihydroxy ester from *Agave* (22) and to mixtures of isomers (5,8). In this study, MS of TMS derivatives of the 4 hydroxyoxo esters and the 4 dihydroxy esters were obtained both of individual isomers and of mixtures of known composition. Figure 3 shows the fragmentation patterns observed for derivatives of isomers substituted at C-9 (**4** and **6**). Oxo esters showed the expected fragmentation resulting from α- or β-cleavage (8,23) and the dihydroxy esters the usual α-cleavage on either side of the carbon, in the middle of the chain, bearing the TMS group (5,22). As noted previously (22), α-cleavage of the bond furthest from the ester group was favored and this tendency increased when the substituent was closer to the ester group. Thus, in the 7,16-di OTMS derivative, the ion m/e 317 has only 20% of the intensity of the ion m/e 231. Another fairly prominent fragment which depended on substituent position, and had m/e 155 for the 9,16-di OTMS derivative (Fig. 3.3) is probably $OHC-(CH_2)_7C\equiv O^+$ resulting from fragmentation occurring after transfer of the terminal TMS group to the carboxyl carbonyl (24). The spectra thus completely confirmed the structures of the isomers.

Mixtures of hydroxyoxo and dihydroxy esters, as TMS ethers, also were analyzed by monitoring and integration of a selected single ion for each compound; the results are in Table I. A diagnostic ion, which was consistently prominent in all 4 isomers, was selected; for oxo esters, it was the ion resulting from McLafferty rearrangement and β-cleavage of the bond on the side of the carbonyl remote from the ester group (in Fig. 3.1, ion with m/e 200

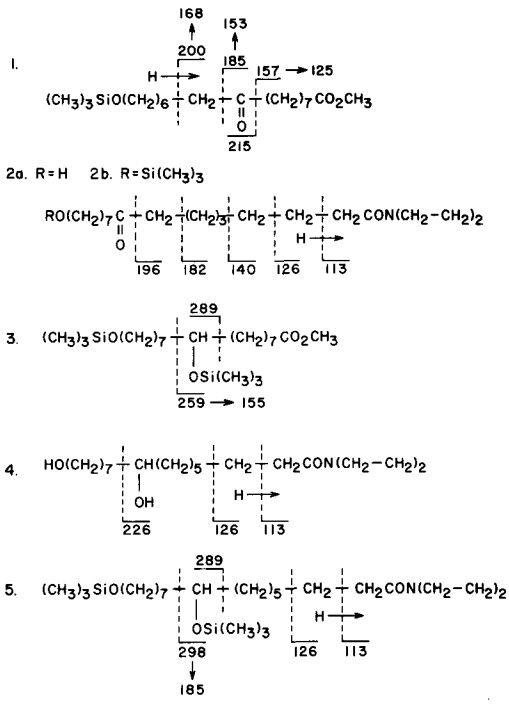

FIG. 3. MS fragmentation of derivatives of hydroxyoxo- and dihydroxyhexadecanoic acids. 1: Methyl 16-hydroxy-9-oxohexadecanoate TMS ether; 2a: 16-hydroxy-9-oxohexadecanoylpyrrolidide; 2b: 16-hydroxy-9-oxohexadecanoylpyrrolidide TMS ether; 3: methyl 9,16-dihydroxyhexadecanoate TMS ether; 4: 9,16-dihydroxyhexadecanoylpyrrolidide; 5: 9,16-dihydroxyhexadecanoylpyrrolidide TMS ether.

from cleavage of 10,11 bond). For di OTMS esters, the ion was that obtained by α-cleavage on the side of the substituted carbon away from the ester group. The results show that the 16-OTMS-7-oxo isomer, from **3**, is considerably overestimated and the 16-OTMS-10-oxo isomer, from **7**, is underestimated. The 7,16-isomer, was also overestimated in analysis of the di OTMS derivatives but not nearly as much.

Because the principal diagnostic ions in spectra of hydroxy and oxo methyl esters are always accompanied by relatively intense ions resulting from loss of 32 amu (23), the use of pyrrolidide derivatives has been investigated as a means of obtaining fewer and more intense diagnostic ions (25).

Pyrrolidides and TMS ether pyrrolidides of the oxygenated acids were examined. In the oxo derivatives (Fig. 3.2), cleavage in the vicinity of the carbonyl group is unaffected by TMS ether formation. As was found during MS investigation of all the isomeric oxooctadecanoylpyrrolidides (25), the principal diagnostic ion is produced by β-cleavage on the amide side

TABLE I

Compositions of Mixtures of Derivatives of Hydroxyoxo- and Dihydroxyhexadecanoic Acids Determined by MS (integration of selected ion profiles)

Hydroxyoxo derivatives	Composition calculated	Composition found[a] Methyl ester TMS ethers	Pyrrolidides
16, 7	26.2	41.7 (172)	19.0 (154)
16, 8	24.6	21.5 (186)	40.1 (168)
16, 9	25.0	20.3 (200)	21.6 (182)
16,10	24.2	16.5 (214)	19.3 (196)
Dihydroxy derivatives		**Methyl ester TMS ethers**	**Pyrrolidides**	**Pyrrolidides TMS ethers**
7,16	25.5	30.9 (231)	29.3 (198)	22.2 (270)
8,16	24.6	23.8 (245)	26.5 (212)	22.9 (284)
9,16	25.4	22.4 (259)	23.5 (226)	25.6 (298)
10,16	24.5	22.9 (273)	20.7 (240)	29.3 (312)

[a]Average of 3 determinations, m/e of selected ion in parentheses.

of the carbonyl (formation of ion m/e 182 in Fig. 3.2). These ions were also selected for integration in mixture analysis but the 16-hydroxy-8-oxo isomer was greatly overestimated. Fission α to the carbonyl occurs to a small extent in pyrrolidides from **8**, **4** and **7** giving a fragment with m/e 14 amu greater than that resulting from β-cleavage but in the 16-hydroxy-7-oxo derivative, α- and β-cleavage products have the same intensity. Thus, the derivative from **3** also gives a prominent α-cleavage ion at m/e 168 which in mixtures contributes to the ion of this m/e formed from the derivative from **8** by β-cleavage. Pyrrolidides of hydroxyoxo acids, while giving fragments suitable for identification of individual isomers, are not suitable for quantitative analysis.

Pyrrolidides of dihydroxy acids, both with free hydroxyls and as TMS ethers, showed strongly amide-directed α-fission (25). Thus, for TMS derivatives, only very weak ions (that at m/e 289 in Fig. 3.5) result from α-cleavage on the amide side of the substituent so that this derivative is less satisfactory for determining structural features at the other end of the chain. An intense ion, m/e 185, was prominent in spectra of all the isomers and probably is:

$$\cdot H_2C\underset{\underset{O}{\|}}{C}\text{-}N(CH_2\text{-}CH_2)_2$$
$$^{+}O\text{-}Si(CH_3)_3,$$

formed by migration of the TMS group on the secondary alcohol oxygen to the amide carbonyl followed by fission of the 2,3 bond. Similar migrations have been described for TMS derivatives of methyl esters (24). This ion also was observed in spectra of the TMS derivatives of the hydroxyoxo pyrrolidides but was less intense, showing that the TMS group could be transferred from one end of the chain to the other (22) but not as readily as from an oxygen situated in the middle of the chain.

During quantitative analysis of the underivatized pyrrolidides, isomers with the substituent closest to the carbonyl gave the greatest response but in the TMS pyrrolidides the reverse response was observed (Table I).

The quantiative estimations show that for hydroxyoxo derivatives, serious errors are introduced only when isomer **3** is present; for dihydroxy derivatives, differences in response are smaller and satisfactory results should be possible using correction factors. Hydroxyoxo esters probably would be best estimated after reduction to dihydroxy esters as has been suggested previously (8).

Reactivity of the Isomers

It was observed that 7,16-dihydroxy methyl esters **5**, which had been kept in crystalline form for 2 years, had undergone extensive intermolecular ester exchange. TLC showed that the product contained a large number of components, presumably polyesters, differing in the number of ester groups. ^{13}C nuclear magnetic resonance (NMR) showed that interesterification had occurred only at the terminal hydroxyl; the secondary hydroxyl was unaffected (26). The diacetate of **5** also polymerized. The reaction was much more pronounced in the 7,16-dihydroxy isomer but preparations of the other isomers showed impurities on TLC (particularly after column chromatography) which could be removed by acid methanolysis. For this reason, all products

were subjected to acid methanolysis before final crystallization. A sample of methyl 16-hydroxyhexadecanoate which had been prepared 18 years previously (27) showed no sign of interesterification, suggesting that the secondary alcohol substituent in **5** may influence reactivity of the carboxyl group. The same reactivity was not observed in the hydroxyoxo esters. It apparently has been assumed that the purpose of the secondary hydroxy groups was to enable crosslinking between chains of polyesters to occur, but our results suggest that it may also facilitate reaction between the carboxyl and the terminal hydroxyl in another molecule.

EXPERIMENTAL

Analytical Procedures

TLC was carried out on precoated plates of Silica Gel 60 (Merck), 0.25 mm thick, which were eluted 3 times with diethyl ether, and spots were developed by charring with 50% H_2SO_4. Under these conditions, R_f values were: methyl 16-hydroxyhexadecanoate, 0.63; methyl 16-hydroxy-7-oxohexadecanoate 0.52; methyl 16-hydroxy-8-oxohexadecanoate, 0.50; methyl 16-hydroxy-9-oxohexadecanoate, 0.49; methyl 16-hydroxy-10-oxohexadecanoate, 0.47; methyl 7,16-dihydroxyhexadecanoate, 0.29; methyl 8,16-dihydroxyhexadecanoate, 0.29; methyl 9,16-dihydroxyhexadecanoate, 0.27; methyl 10,16-dihydroxyhexadecanoate, 0.22. Oxohydroxy esters were not separated from neighboring isomers but mixtures of 9,16- and 10,16-dihydroxy esters gave 2 separate spots.

Silica gel (Biosil A, 200-400 mesh, Bio-Rad, Richmond, CA), activated at 110 C, was employed for column chromatography.

TMS derivatives for GC-MS were prepared by treatment with N,O-bis(trimethylsilyl)-acetamide in CH_2Cl_2 solution. To obtain pyrrolidides, 5 mg of methyl esters were dissolved in a mixture of 1 g of pyrrolidine and 0.1 g of acetic acid and kept at 25 C for 18 hr. The solution was poured into water, extracted with CH_2Cl_2, washed with 1 N HCl and with water and dried (Na_2SO_4). Removal of the solvent gave derivatives which did not require further purification.

A Finnigan model 3300 instrument with an Incos model 2000 data system was used for GC-MS analysis; the ionization voltage was 70 eV. The glass column was 1.7 m × 2 mm id, packed with 1.5% Dexsil 300 on 80-100 mesh acid washed and silanized Chromosorb W. When TMS derivatives were analyzed, temperature was programmed from 175 to 300 C at 4 C/min. Pyrrolidides with free hydroxyl groups were insufficiently volatile for GC analysis and were inserted into the spectrometer with a probe inlet. In analyzing mixtures of isomeric derivatives, selected ion profiles at 4 appropriate masses, in order from 10-substituted to 7-substituted, were integrated. For TMS ethers of hydroxyoxo methyl esters: ions with m/e 214, 200, 186, 172; of dihydroxy methyl esters: 273, 259, 245, 231; for hydroxyoxo pyrrolidides: 196, 182, 168, 154; for dihydroxy pyrrolidides: 240, 226, 212, 198; and for TMS ethers of dihydroxy pyrrolidides: 312, 298, 284, 270 were examined.

Methyl 16,17-Dihydroxy-7-oxoheptadecanoate (1)

7-Oxo-16-heptadecenoic acid was prepared as previously described (10) and converted to the methyl ester with methanolic HCl (5%). Methyl 7-oxo-16-heptadecenoate had bp/0.1 mm 131-134 C, after crystallization from hexane the mp was 32.5-33.5 C.

Anal. calcd for $C_{18}H_{32}O_3$: C, 72.92; H, 10.88; found: C, 72.75; H, 10.94. Epoxide route: a solution of 30 g (0.1 mol) of the oxo ester just described and 21.84 g (0.125 mol) of *m*-chloroperbenzoic acid in 350 ml of CH_2Cl_2 was kept at 25 C for 7 hr. The solution was then diluted with 300 ml of CH_2Cl_2 and washed with Na_2SO_3, $NaHCO_3$ and NaCl solutions and dried over Na_2SO_4. After removal of solvent, crude epoxide was dissolved in 250 ml of acetic acid and refluxed for 4 hr. The reagent was taken off under reduced pressure and the residue refluxed with methanolic HCl (5%) for 3 hr. The solution was poured into 20% NaCl solution and the product extracted with $CHCl_3$; after removal of the solvent, crystallization from acetone yielded 20.6 g (60%) dihydroxy-oxo ester **1**, mp 73-74 C.

Anal. calcd for $C_{18}H_{34}O_5$: C, 65.42; H, 10.37; found: C, 65.51; H, 10.32. Hydroxylation route: hydrogen peroxide (30%, 8 g) was added to 20 g (0.0676 mol) of methyl 7-oxo-heptadecenoate in 100 ml of formic acid (97%) and the solution was stirred for 7 hr at 37 C. The reagents were removed at 70 C and 12 mm and the residue refluxed for 1.5 hr in methanol containing 2.5% HCl. The product was worked up as just described and crystallized from acetone giving 16.85 g (76%) of **1**.

Methyl 16-hydroxy-7-oxohexadecanoate (3)

A solution of 6.6 g (0.02 mol) of ester **1** in 100 ml of acetic acid was stirred at 20 C for 15 min during addition of 13.3 g (0.03 mol) lead tetraacetate and stirring was continued for

1.5 hr. After addition of 300 ml of benzene, the solution was poured into 700 ml of water containing 1 g of glycerol. The benzene layer was separated and the water reextracted with 300 ml of benzene; combined extracts were washed with water, with $NaHCO_3$ solution and with water and dried (Na_2SO_4). Solvent was removed at 25 C, leaving 6.0 g of white solid. Sodium borohydride (1.52 g, 0.04 mol) was suspended in 250 ml of benzene and 7.2 g (0.12 mol) of acetic acid was added (13). The mixture was refluxed for 15 min when most of the borohydride had dissolved; a solution of the oxoaldehyde already mentioned in 150 ml of benzene was added and reflux continued for 1.5 hr. The solution was cooled, washed with water and dried (Na_2SO_4).

The crude product was chromatographed on 200 g of silica gel, elution with hexane/acetone (9:1) gave 1.11 g of a mixture of by-products and hydroxy-oxo ester followed by 4.16 g (69%) of hydroxyoxo ester **3**. After refluxing with methanolic HCl (%) for 18 hr to break down any polyesters present, and recovery 3 was crystallized from acetone giving large leaflets, mp 57-58 C; MS of methyl ester TMS ether m/e (rel intensity) M^+ missing, 357 M-15 (7), 227 (6), 185 (4), 172 (13), 157 (13), 140 (16), 125 (30), 103 (30), 75 (100); of pyrrolidide 339 M^+ (2), 196 (4), 168 (20), 154 (19), 140 (7), 126 (35), 113 (100); of pyrrolidide TMS ether 411 M^+ (0.6), 396 (6), 185 (12), 168 (17), 154 (19), 140 (6), 126 (3), 113 (100), 103 (6).

Anal. calcd for $C_{17}H_{32}O_4$: C, 67.96; H, 10.74; found: C, 67.96; H, 10.75.

Methyl 7,16-Dihydroxyhexadecanoate (5)

Hydroxyoxo ester **3** (1.01 g, 0.0034 mol) was dissolved in 30 ml of methanol, 0.16 g (0.004 mol) of sodium borohydride added and the solution was stirred at 20 C for 90 min. After acidification with 1 ml of acetic acid and dilution with 200 ml of 20% NaCl solution, product was extracted with chloroform. Crystallization from acetone gave dihydroxy ester **5** (80% yield) small leaflets, mp 53-54 C; MS of methyl ester di TMS ether m/e (rel intensity) M^+ missing, 431 M-15 (1), 317 (5), 231 (26), 199 (4), 129 (18), 127 (17), 103 (20), 73 (100); of pyrrolidide 341 M^+ (0.6), 198 (11), 168 (4), 140 (3), 126 (33), 113 (100); of pyrrolidide di TMS ether M^+ missing, 470 M-15 (7), 270 (27), 198 (11), 185 (18), 169 (9), 140 (5), 126 (32), 113 (92), 103 (20), 73 (100).

Anal. calcd for $C_{17}H_{34}O_4$: C, 67.51; H, 11.33; found: C, 67.32; H, 11.27.

6-Heptenylmalonic acid

4-Pentene-1-ol (28) was converted to mesylate by the method described previously (12,-29) and then to 4-pentenylmalonic acid by the Spener and Mangold method (30). Crystallization from benzene gave acid with mp 89-91 C, lit. (31) mp 87 C. Decarboxylation yielded 6-heptenoic acid, bp 98 C/5 mm. The acid was reduced to alcohol and the described route followed to 6-heptenylmalonic acid, crystals from benzene, mp 100-102 C, lit. (31) mp 90-91 C.

Methyl 9-Oxo-16-heptadecenoate

Reaction between the sodium derivative of the tetrahydropyranyl ester of 6-heptenylmalonic acid and methyl 8-chloroformyloctanoate by the method previously described (11,12) gave crude metyl oxo ester. After hydrolysis with KOH, crystallization from acetone gave 9-oxo-16-heptadecenoic acid (71% yield), mp 67.5-68.5 C.

Anal. calcd for $C_{17}H_{30}O_3$: C, 72.30; H, 10.71; found; C, 72.41; H, 10.42.

Acid was reconverted to methyl ester, with methanolic HCl, and a portion crystallized from hexane giving pure methyl 9-oxo-16-heptadecenoate, mp 37.5-38.5 C.

Anal. calcd for $C_{18}H_{32}O_3$: C, 72.92; H, 10.88; found: C, 72.73; H, 11.11.

Methyl 16,17-Dihydroxy-9-oxoheptadecanoate (2)

This ester was prepared (59% yield) from methyl 9-oxo-16-heptadecenoate by the epoxide route already described. The product was crystallized from acetone, mp 72-73 C.

Anal. calcd for $C_{18}H_{34}O_5$: C, 65.42; H, 10.30; found: C, 65.19; H, 10.24.

Methyl 16-Hydroxy-9-oxohexadecanoate (4)

Lead tetraacetate oxidation, followed by sodium triacetoxyborohydride reduction of 5 g of **2** as already described gave crude hydroxy-oxo ester **4**. The product was purified by column chromatography and methanolic HCl treatment giving 2.7 g (59%) of **4**, after acetone crystallization the mp was 55-56 C; MS of TMS ether m/e (relative intensity) M^+ missing 357 M-15 (5), 215 (5), 200 (6), 185 (7), 168 (3), 157 (7), 153 (5), 140 (16), 125 (38), 75 (89), 55 (100); of pyrrolidide 339 M^+ (0.9), 196 (4), 182 (18), 140 (4), 126 (21), 113 (100); of pyrrolidide TMS ether 411 M^+ (0.4), 396 M-15 (4), 196 (3), 185 (15), 182 (16), 140 (4), 126

(19), 113 (100).

Anal. calcd for $C_{17}H_{32}O_4$: C, 67.96, H, 10.74; found: C, 68.16; H, 11.01.

Methyl 9,16-Dihydroxyhexadecanoate (6)

Reduction of hydroxyoxo ester **4** as described yielded dihydroxy ester **6**. The product was purified by column chromatography, methanolic HCl treatment and crystallization from acetone, mp 59-60 C; MS of di TMS ether m/e (relative intensity) M^+ missing, 431 M-15 (0.8), 289 (12), 259 (24), 155 (26), 129 (26), 109 (39), 103 (22), 73 (100); of pyrrolidide 341 M^+ (0.4), 226 (8), 140 (3), 126 (25), 113 (100); of pyrrolidide di TMS ether M^+ missing, 470 M-15 (4), 298 (25), 289 (2), 185 (30), 140 (4), 125 (21), 113 (100), 103 (16).

Anal. calcd for $C_{17}H_{32}O_4$: C, 67.51; H, 11.33; found: C, 67.66; H, 11.38.

Methyl 16-Hydroxy-8-oxohexadecanoate (8)

Dihydroxy ester **2** (10.3 g, 0.031 mol) was acetylated with 50 ml acetic anhydride and 50 ml pyridine at 25 C for 18 hr; the reagents were removed at 70 C and 0.1 mm. The residual waxy solid was dissolved in 150 g of 2-ethyl-2-methyl-1,3-dioxolane, 0.15 g *p*-toluenesulfonic acid was added and the mixture was refluxed for 4 days. During the last 3 days, part of the reagent was distilled off but GLC indicated only 25% ketal formation. The solution was diluted with 300 ml of benzene and washed with $NaHCO_3$ solution and with water and dried (Na_2SO_4); removal of the solvent gave 13 g of oil. The partially ketalized ester was dissolved in 600 ml of dimethoxyethane (distilled from $LiAlH_4$), 6 g (16 mol) of $LiAlH_4$ added and the suspension refluxed for 18 hr. The reaction mixture was decomposed by addition of 6 ml of water, 6 ml 15% aqueous NaOH and 18 ml of water, and then boiled and filtered. Solvent was removed from the filtrate and used to reextract the precipitate by refluxing for 10 min; after this procedure was repeated one further time, the total of recovered crystalline reduction product was 9.9 g.

The product was dissolved in 150 ml acetic acid and oxidized with 15 g of lead tetraacetate as described to give 8.3 g of solid cleavage product. This product was acetylated in 50 ml pyridine with 50 ml acetic anhydride for 3 hr at 25 C; the reagents were then removed at 30 C and 0.1 mm. The acetylated aldehyde was dissolved in 100 ml of acetic acid and 2.6 g (0.026 mol) of chromium trioxide in 5 ml water and 25 ml acetic acid was added with stirring at 20 C during 30 min. After a further 30 min, the mixture was poured into 600 ml of 4 N H_2SO_4 and the product extracted with $CHCl_3$. After solvent removal and reflux with methanolic HCl for 18 hr and work-up as usual, 7.7 g of crude hydroxyoxo and dihydroxy esters were obtained. This product was chromatographed on 200 g silica gel, elution with hexane/acetone (9:1) gave 1.2 g (13%) of 16-hydroxy-8-oxo ester **8** and with hexane/acetone (4:1) gave 2.9 g (31%) of 8,16-dihydroxy ester **10**.

The hydroxyoxo ester was again treated with methanolic HCl and crystallized from acetone, mp 57.5-59.5; MS of TMS ether m/e (relative intensity) M^+ missing, 357 M-15 (7), 187 (6), 186 (6), 171 (17), 154 (14), 144 (11), 143 (12), 139 (21), 129 (20), 111 (38), 103 (27) 75 (100); of pyrrolidide 339 M^+ (1), 207 (5), 182 (6), 168 (24), 140 (5), 126 (21), 113 (100); of pyrrolide TMS ether 411 M^+ (0.5), 396 M-15 (5), 185 (12), 182 (5), 168 (21), 140 (4), 126 (20), 113 (100).

Anal. calcd for $C_{17}H_{32}O_4$: C, 67.96; H, 10.74; found: C, 67.94; H, 10.61.

Methyl 8,16-Dihydroxyhexadecanoate (10)

Dihydroxy ester obtained as described was treated with methanolic HCl and crystallized from acetone, mp 60-61 C; MS of di TMS ether m/e (rel intensity) M^+ missing, 431 M-15 (1), 303 (9), 245 (27), 141 (20), 129 (20), 103 (17), 73 (100); of pyrrolidide 341 M^+ (0.5), 212 (10), 140 (5), 126 (27), 113 (100); of pyrrolidide di TMS ether M^+ missing, 470 M-15 (6), 303 (1), 284 (28), 185 (24), 140 (5), 129 (17), 126 (28), 113 (86), 103 (17), 73 (100).

Anal. calcd for $C_{17}H_{34}O_4$: C, 67.51; H, 11.33; found: C, 67.54; H, 11.29.

Methyl 10,16-Dihydroxyhexadecanoate (9)

Dihydroxy ester **1** was reduced with $LiAlH_4$ in dimethoxyethane, cleaved with $Pb(OAc)_4$, acetylated and oxidized as already described. After chromatographic purification, the product was crystallized from acetone, mp 63-64 C; MS of di TMS ether m/e (rel intensity) M^+ missing, 431 M-15 (0.5), 275 (11), 273 (22), 169 (15), 147 (12), 129 (26), 103 (31), 73 (100); of pyrrolidide M^+ 341 (0.3), 240 (7), 140 (3), 126 (26), 113 (100); of pyrrolidide di TMS ether M^+ missing, 470 M-15 (3), 312 (23), 275 (1), 185 (27), 140 (3), 126 (20), 113 (100), 103 (21).

Anal. calcd for $C_{17}H_{34}O_4$: C, 67.51; H, 11.33; found: C, 67.65; H, 11.22.

Methyl 16-Hydroxy-10-oxohexadecanoate (7)

Dihydroxyester **9** (0.5 g, 0.0017 mol), was

dissolved in 30 ml of CH_2Cl_2 containing 0.1 g (0.006 mol) of hexamethylphosphoramide, 18.5 ml of an 8% aqueous solution of $NaHCO_3$ added and the mixture stirred vigorously with cooling to 0 C. A solution of 0.41 g (0.0025 mol) of bromine in 14 ml of CH_2Cl_2 was added by drops during 25 min (16). The mixture was stirred for a total of 65 min, by which time the bromine color had almost disappeared. The aqueous layer was separated and the CH_2Cl_2 layer washed 3 times with water and dried (Na_2SO_4). Solvent was removed and the residue chromatographed; elution with hexane/acetone (9:1) gave 0.23 g (46%) of 7. The hydroxyoxo ester was crystallized from hexane, mp 58-59 C; MS of TMS ether m/e (rel intensity) M^+ missing, 357 M-15 (7), 214 (8), 201 (5), 199 (6), 185 (7), 159 (17), 143 (15), 139 (16), 126 (32), 103 (33), 75 (100); of pyrrolidide 339 M^+(2), 210 (3), 196 (21), 182 (2), 168 (3), 140 (5), 126 (25), 113 (100); of pyrrolidide TMS ether M^+ missing, 396 M-15 (3), 196 (13), 185 (16), 140 (4), 126 (19), 113 (100).

Anal. calcd for $C_{17}H_{32}O_4$: C, 67.96; H, 10.74; found: C, 68.01; H, 10.99.

Spontaneous Polymerization of Methyl 7,16-Dihydroxyhexadecanoate

After crystalline methyl 7,16-dihydroxyhexadecanoate had been kept in a vial for 2 years, the mp was 77-82 C; TLC showed no material with the same R_f as the original dihydroxy ester but showed at least 6 components with smaller R_f values. The ^{13}C NMR spectrum ($CDCl_3$) showed signals at 24.95 (C-3 polyester), 25.02 (C-3 methyl ester), 25.34 (C-5), 25.68 (C-9), 25.79 (C-14 methyl ester), 25.97 (C-14 polyester), 28.69 (C-15 polyester), 29.24-29.72 (unassigned), 32.76 (C-15 methyl ester), 34.07 (C-2 methyl ester), 34.38 (C-2 polyester), 37.24 (C-6), 37.51 (C-8), 51.41 (OCH_3), 62.96 (C-16 methyl ester) and 64.49 (C-16 polyester). The ^{13}C NMR spectrum of methyl 16-hydroxyhexadecanoate which had been stored for 18 years had signals at 25.00 (C-3), 25.82 (C-14), 29.19-29.66 (unassigned), 32.85 (C-15), 34.15 (C-2) and 62.97 (C-16). A sample of methyl 7,16-diacetoxyhexadecanoate which had originally been distilled to a clear liquid had solidified to an amorphous solid, insoluble in $CHCl_3$, after 2 years of storage. Both polymerized dihydroxy and diacetoxy compounds were completely reconverted to methyl 7,16-dihydroxyhexadecanoate by 18 hr of reflux with methanolic HCl.

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Absorption and Distribution of Jojoba Wax Injected Subcutaneously into Mice

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ABSTRACT

^{14}C-Labeled jojoba wax was injected subcutaneously into mice and ^{14}C was determined 1-90 days after application in several internal organs, in the skin and in the lipids extracted from the carcass. A control group of mice was similarly treated with triolein. The major part of the injected lipids was not absorbed or metabolized. Some label was found in the organs examined, but there was no accumulation of labeled lipids with time. About 20% of label derived from triolein was found in polar lipids, whereas only 2-4% of that derived from jojoba wax was found in this fraction. There was some (1-5%) incorporation of the label of jojoba wax into body triglycerides.

INTRODUCTION

Jojoba wax is a natural liquid wax (mp 7 C) derived from the seeds of the shrub *Simmondsia chinensis* (Link) Schneider (jojoba). This evergreen shrub, which is indigenous to Southern California, Arizona and northern Mexico, is now being domesticated in Israel's Negev desert.

The wax consists of esters of straight chain monoethylenic acids, mainly eicos-11-enol and docos-11-enol (1). It has been reported to have potential as an ingredient in cosmetic preparations (2,3) and has been shown to be safe for cosmetic use by a series of acute toxicity studies (3).

We have initiated a series of efficacy studies on the wax as a skin emollient in cosmetic preparations and as a component in medications for diseased skin. However, before further studies can be performed on the effects of the wax for external use, more information is required on the degree of systemic absorption of the wax and its fate in the body. Is it deposited or excreted? Does it pass through the system unchanged or is it metabolized? The studies reported in this paper were designed to answer these questions. Radioactively labeled wax was injected subcutaneously (SC) into mice and the distribution of the label was determined at intervals up to 3 months after application.

EXPERIMENTAL PROCEDURES

Animals

Five-week-old albino mice, each weighing 25-30 g, were used. The mice were housed in groups of 2-5/cage and were fed ad libitum.

^{14}C-Labeled jojoba wax. Randomly labeled jojoba wax was obtained by exposure of fruiting branches of the shrub to $^{14}CO_2$ fluxes as described previously (4). Mature seeds from these branches were picked, lyophilized and extracted with petroleum ether. The extract was treated with 1% bleaching earth, and the solvent was evaporated. The composition of the wax and the distribution of the label among its lipid components, as determined by thin layer chromatography (TLC) and spot scintillation counting, are shown in Table I.

TABLE I

TLC Characterization of Bleached ^{14}C-Labeled Jojoba Wax[a]

R_f	^{14}C Distribution (%)	Standards
0.00-0.16	0.1	Glycolipids and phospholipids
0.16-0.23	0.5	Sterols
0.23-0.27	0.5	Fatty alcohols
0.27-0.36	0.7	Fatty acids
0.36-0.55	0.3	
0.55-0.76	0.1	
0.76-0.90	0.1	Triolein
0.90-1.00	97.7	Wax esters

[a]The plates were developed in petroleum ether (60-80 C)/ethyl ether/acetic acid, 80:20:1, v/v/v.

[^{14}C] Triolein. Triolein (glyceryl tri[1-^{14}C]-oleate, specific activity [sp act] 30-60 mCi/mmol, The Radiochemical Centre, Amersham, England) was supplied in benzene solution. The solution was diluted with refined olive oil to a sp act of 1.37 μCi/g, and the benzene was evaporated.

Injection of the Lipids

Labeled wax or labeled triolein was injected SC into the rear right leg of each mouse, at the amount and sp act specified in each experiment. The leg, being as far as possible from the internal organs, was chosen as the injection site so that contamination of the internal organs during autopsy could be minimized. In experiment 3, the wax was injected SC into the neck, since at that site it is easier to ensure that the injection will be SC and not intramuscular. The results, however, do not seem to be affected by the injection site.

Autopsy Procedure

The mice were killed by anesthetization with ethyl ether, since a preliminary study showed that the method of killing–cervical fracture or ether anesthetization–did not affect the absorption and distribution of the injected lipid. Immediately after death, a piece of skin (ca. 400 mg) was taken from a site as far as possible from the injection location, the organs to be studied were removed and the connective tissue was trimmed off. Each sample of organ or skin was washed with cold 0.25 M sucrose solution, blotted dry, weighed and frozen.

Determination of ^{14}C in Whole Organs and Skin

The organs or the skin samples were digested with Soluene 350 (Packard), 1 ml of Soluene/100-200 mg of tissue. The digest was then cooled and decolorized after the addition of isopropanol and 30% H_2O_2, and neutralized with glacial acetic acid (40 μl/1 ml of digest in Soluene, according to the method recommended by the manufacturer). The digest was then transferred to a vial containing 10 ml of scintillation solution, and radioactivity was counted in a Packard scintillation counter model 3380. The results are expressed as wax or triolein equivalents in μg (although ^{14}C can exist in the organ either in the form injected or as its metabolites).

Lipid Extraction

The frozen organs were ground with a pestle and mortar at 0-4 C, and the lipids were extracted by the Folch et al. procedure (5). The carcass (after the internal organs studied had been removed) was frozen and lyophilized for 48 hr. The lipids were then extracted with petroleum ether (40-60 C) in a Soxhlet apparatus for 48 hr. The extracted lipids were dissolved in toluene and characterized by TLC. Radioactivity was counted in samples before and after TLC.

Lipid Analysis by TLC

TLC was performed on silica-gel-coated plates, Kieselgel 60F254, Merck. The plates were developed with petroleum ether (60-80 C) ethyl ether/acetic acid (80:20:1 or 90:10:1, v/v/v). The 80:20:1 solvent system gave better separation between fatty acids, fatty alcohols and triglycerides, and the 90:10:1 between triglycerides and wax esters.

RESULTS

Absorption and Distribution of the Wax As a Function of Time

Two experiments were carried out. In experiment 1, 90 ± 10 mg of ^{14}C-labeled jojoba wax with sp act 1.14 μCi/g were injected SC into each of 24 male mice. In a control group of the same size, each mouse was injected with 100 ± 20 mg [^{14}C] triolein with sp act 1.37 μCi/g. Triolein was used as the control, since the metabolism of triglycerides in the animal body has already been studied, in comparison to the metabolism of jojoba wax, on which no data exist. Six mice from each group were killed after 1, 8, 15 and 23 days. The label in the lipid and aqueous fractions of the liver and the brain, and the total radioactivity in the testis, skin and carcass (including the injection area) were determined (Table II). Only a small portion of the injected lipid, whether jojoba wax or triolein, was found in the organs studied even in the mice killed after 23 days. The label was distributed in the organs studied after as short a time as one day after application. Label was also found in other internal organs, but the recorded counts were very low, and results therefore are not reported. For both the wax and the triolein, most of the label was recovered in the lipid fractions, but traces were also found in the aqueous fractions. The radioactivity in the liver lipids decreased over the 23 days after injection, from an amount equivalent to ca. 57 ± 16 μg of jojoba wax to 15 ± 7 μg; the amount remaining in the tissue corresponds to 0.03 μmol of jojoba wax esters. The content of triolein in the liver and its change with time were in the same range as those of jojoba wax. In the brain lipids, there was a similar drop with time in the ^{14}C derived

TABLE II

Distribution of ^{14}C As a Function of Time after Subcutaneous Injection of Labeled Jojoba Wax or Labeled Triolein

	Distribution of ^{14}C in the organs (μg/organ)[a]									
	Experiment 1								Experiment 2	
	[^{14}C]Jojoba wax (μg)[a]				[^{14}C]Triolein (μg)[a]				[^{14}C]Jojoba wax (μg)[a]	[^{14}C]Triolein (μg)[a]
Tissue or organ	1 d	8 d	15 d	23 d	1 d	8 d	15 d	23 d	90 d	90 d
Liver lipids	57 ± 16	23 ± 5	22[b] ± 1	15 ± 7	43 ± 6	37 ± 10	26 ± 4	19 ± 2	18 ± 6	56 ± 12
Liver aqueous extract	9 ± 3	1 ± 0	6 ± 5	4 ± 1	5 ± 1	2 ± 1	2 ± 1	2 ± 1	17 ± 9	9 ± 2
Brain lipids	108 ± 46	21 ± 7	14 ± 6	9 ± 4	5 ± 2	9 ± 1	9 ± 3	9 ± 1	16 ± 6	17 ± 4
Brain aqueous extract	6 ± 2	4 ± 1	2 ± 1	0	2 ± 1	1 ± 0	12 ± 8	14 ± 10	3 ± 2	3 ± 1
Skin (400 mg)	26 ± 13	9 ± 4	11 ± 2	19 ± 5	9 ± 2	29 ± 8	16 ± 2	13 ± 5	4 ± 1	65 ± 17
Testis	11 ± 4	11 ± 2	11 ± 4	13 ± 5	15 ± 6	30 ± 8	25 ± 6	20 ± 3	3[c] ± 2	25[c] ± 2
Total recovered from examined portions	217 ± 84	69 ± 19	66 ± 19	60 ± 22	79 ± 18	108 ± 28	90 ± 24	77 ± 22	61 ± 26	175 ± 38
Total body lipids X 10^3	100 ± 4	65 ± 12	78 ± 8	ND	99 ± 4	ND	87 ± 11	ND	80 ± 16	100 ± 23

Data in the table are given for 1 out of 4 replicate experiments, containing 6 and 10 mice in each group of experiment 1 and 2, respectively. Injection dose–90 ± 10 mg jojoba wax of 1.14 μCi/g or 100 ± 20 mg triolein of 1.37 μCi/g.

d = days after injection; ND = not determined.

[a]The amount of lipid present in an organ (or tissue) was calculated as μg (± SE) from the sp act of the injected lipid and the counts of that organ, ^{14}C in organ (dpm) ÷ ^{14}C in injected lipid (dpm/μg).

[b]The reported value is the mean of 5 mice livers; the sixth value was exceptionally high.

[c]This result is the mean for 5 male mice. The mean value of labeled lipids in the ovaries of 5 female mice was 2 ± 1 μg/ovary for the jojoba group and 82 ± 27 μg/ovary for the triolein group.

from the wax (from a value of 108 ± 46 μg wax equivalents 1 day after application to a value of 9 ± 1 μg at the 23rd day), whereas the [^{14}C]-triolein content was low (9 μg) over the whole period (Table II).

In experiment 2, 2 groups consisting of 5 male and 5 female mice each were injected as in experiment 1. All the mice were killed 90 days after the lipid injection, and the radioactivity was determined as in experiment 1. It is notable that label was still found in the internal organs 90 days after injection in both the jojoba-wax-treated and triolein-treated animals. At this time, however, the absorption and distribution patterns of the label in the jojoba-treated mice differed from those in the triolein-injected animals, i.e., a higher content of label was found in the liver lipid, skin and testis of the triolein-injected animals (Table II).

The values of ^{14}C in carcass lipids indicate that most of the injected label, whether wax or triolein, was retained in the carcass even after periods as long as 90 days (Table II).

Absorption and Distribution of the Wax As a Function of Dose

When the 2 experiments just reported were completed, we obtained a new crop of jojoba wax of a similar chemical composition to that used in experiments 1 and 2 but with a higher sp act. This advantage enabled us to study, also, the absorption of a smaller dose of wax than the doses used before and to determine the label quantitatively in additional internal organs. Three doses of 9, 23 and 120 mg wax were injected SC into the dorsal neck of 3 groups of 7 male mice each. The distribution of the label was determined, on the basis of an organ or of g wet weight, 8 days after application (Table III). The lowest level of radioactivity was found in the organs of animals injected with the lowest dose (9 mg). No significant differences were, however, found between animals injected with the 2 higher doses. ^{14}C concentration as expressed on a wet weight basis is in the same range in the different organs, with lower quantities in the liver and higher in the skin and in the epididymal fat tissue.

TLC Characterization of Carcass Lipids

The radioactivity TLC profile of carcass lipids of the jojoba-injected group shows that most of the injected ^{14}C remained in the body in the lipid form in which it was injected, i.e., as wax esters (spot 8, Table IV). A high percentage of ^{14}C also was found in spot 7 (Table IV), which corresponds to the location of triglycerides. In order to check whether this high percentage might be a result of incomplete chromatographic separation between the wax esters and the triglycerides, we performed another series of TLC separations in a solvent system (petroleum ether/ethyl ether/acetic acid, 90:10:1, v/v/v) that allows a better separation of these lipid groups (Table V). The results show that, indeed, most of the ^{14}C remained in the form of wax esters, i.e., 97% at 1 day and ca. 90% at 8, 23 and 90 days after application, but the remaining ^{14}C was incorporated into the various carcass lipids—mainly neutral lipids, such as triglycerides and fatty acids.

Similarly, in the triolein group, ca. 75-80% of the ^{14}C was in the form of triglycerides (spot 7, Table IV). At this stage, we could not distinguish between the ^{14}C in the original triolein and the triglycerides. A considerable

TABLE III

Distribution of ^{14}C in the Body, Eight Days after Subcutaneous Injection of Labeled Jojoba Wax As a Function of the Injected Dose

Organ or tissue	Wax equivalents (μg[a]/organ)			Wax equivalents (μg[a]/fresh wt)		
	Injected dosage (mg)					
	9	23	120	9	23	120
Liver	3.7 ± 0.7	8.2 ± 0.7	7.7 ± 0.4	2.2 ± 0.4	4.8 ± 1.0	4.5 ± 0.9
Brain	5.2 ± 3.5	6.8 ± 3.0	2.3 ± 1.0	16.1 ± 10.9	17.5 ± 7.6	12.2 ± 5.4
Testis	0.5 ± 0.1	2.8 ± 0.5	1.2 ± 0.4	3.0 ± 0.7	16.5 ± 2.3	14.4 ± 5.4
Lungs	2.3 ± 1.0	7.6 ± 1.9	4.5 ± 2.0	6.2 ± 1.2	34.4 ± 9.1	31.0 ± 12.4
Heart	0.9 ± 0.2	3.1 ± 0.6	2.8 ± 1.0	7.0 ± 1.7	20.7 ± 4.7	13.2 ± 4.3
Spleen	1.0 ± 0.2	2.7 ± 0.7	2.4 ± 1.0	9.2 ± 1.9	24.5 ± 7.4	22.3 ± 10.0
Kidney	1.4 ± 0.3	4.4 ± 1.1	3.7 ± 0.9	6.5 ± 1.7	20.0 ± 5.1	17.1 ± 4.0
Skin	–	–	–	20.1 ± 4.3	63.0 ± 13.0	28.5 ± 5.2
Epididymal fat	–	–	–	61.5 ± 12.6	162.1 ± 37.5	128.0 ± 4.4
Carcass lipids X 10^3	5.5 ± 0.6	16.1 ± 3.0	ND	–	–	–

[a]As in Table II.

TABLE IV

Radioactivity TLC Profile of Body Lipids at Fixed Time Intervals after ^{14}C-Lipid Injection[a]

Spot no.	R_f	Incorporation of ^{14}C into lipid fraction (%)								Lipid standards
		Experiment 1						Experiment 2		
		[^{14}C]Jojoba wax			[^{14}C]Triolein			[^{14}C]Jojoba wax	[^{14}C]Triolein	
		1 d	8 d	23 d	1 d	8 d	23 d	90 d	90 d	
1	0.00-0.02	0.2 ± 0.1	0.6 ± 0.4	0.6 ± 0.1	5.5 ± 0.3	4.1 ± 0.4	3.8 ± 0.6	0	3.0 ± 0.9	Phospholipids
2	0.02-0.04	1.2 ± 0.2	0.6 ± 0.4	1.1 ± 0.2	5.6 ± 0.1	3.6 ± 0.5	4.5 ± 0.5	0	2.3 ± 0.8	
3	0.04-0.08	1.0 ± 0.5	1.0 ± 0.2	0.7 ± 0.3	4.8 ± 0.5	3.4 ± 0.8	2.6 ± 0.6	0.9 ± 0.5	3.0 ± 0.7	Sterols
4	0.08-0.12	0.6 ± 0.2	0.7 ± 0.2	0.9 ± 0.1	1.1 ± 0.1	0.8 ± 0.3	0.9 ± 0.2	2.7 ± 1.6	1.3 ± 0.5	
5	0.12-0.35	0.3 ± 0.2	1.3 ± 0.4	2.2 ± 0.4	0	9.1 ± 0.6	6.5 ± 0.8	2.0 ± 0.9	9.1 ± 2.1	Fatty acids
6	0.35-0.55	0	0.2 ± 0.1	0.3 ± 0.2	0	1.0 ± 0.2	2.2 ± 0.6	0.8 ± 0.3	0.5 ± 0.4	
7	0.55-0.85	28.9 ± 5.0	12.7 ± 5.7	19.2 ± 6.0	82.4 ± 0.1	74.9 ± 2.2	78.9 ± 1.0	6.1 ± 1.9	79.8 ± 2.2	Triglycerides
8	0.85-0.95	67.8 ± 6.0	82.9 ± 7.7	75.0 ± 6.0	0.6 ± 0.2	3.1 ± 2.1	0.6 ± 0.3	87.5 ± 4.2	1.0 ± 0.8	Wax esters, Cholesterol esters

[a]The results are the means ± SE of 6 animals in experiment 1 and of 10 animals in experiment 2. Petroleum ether/ethyl ether/acetic acid, 80:20:1, v/v/v, was used as the developing solvent.

TABLE V

Radioactivity TLC Profile of Body Lipids at Fixed Time Intervals after ^{14}C-Lipid Injection[a]

R_f	Incorporation of ^{14}C into lipid fraction (%)								Lipid standards
	Experiment 1						Experiment 2		
	[^{14}C]Jojoba wax			[^{14}C]Triolein			[^{14}C]Jojoba wax	[^{14}C]Triolein	
	1 d	8 d	15 d	1 d	8 d	15 d	90 d	90 d	
0.00-0.03	0.1	0.7	0.6	6.2	6.1	5.5	1.3	6.8	
0.03-0.25	2.1	3.5	3.3	13.8	9.3	14.5	2.8	13.3	Fatty acids
0.25-0.29	0.1	0.1	0.6	0.9	1.5	1.6	1.1	1.3	
0.29-0.59	1.4	3.5	5.5	78.5	80.8	77.5	5.7	75.7	Triglycerides
0.59-0.85	96.9	91.7	89.6	0.4	2.0	0.8	88.9	2.8	Wax esters

[a]The developing solvent was petroleum ether/ethyl ether/acetic acid, 90:10:1, v/v/v.

difference was found between jojoba wax and triolein in the incorporation of label into polar lipids. The carcass lipids of the mice injected with the jojoba wax contained a very small percentage of the label, 0-2%, in polar lipids such as phospholipids (spots 1-3, Table IV). In contrast, the incorporation of ^{14}C into these lipids in the carcass of triolein-injected mice was much higher—ca. 8-10% (spots 1-3, Table IV).

DISCUSSION

Several workers (6,7) have shown that when triglycerides, fatty acids or fatty alcohols were injected intravenously, they were rapidly removed from the plasma, i.e., in 10 min 60% of the triglycerides or fatty acids injected into the rats were cleared from the blood (6), and a large proportion could be recovered from the liver. Gozlan-Devillierre et al. (8) found that when stearic acid was injected SC into mice, traces reached the brain within a few hr and were incorporated into the brain lipids.

In our study, quite large quantities of wax were injected because of the low sp act of the labeled wax available to us; in this aspect, our experiments differ from those just mentioned. As expected, only a small fraction of the wax was absorbed (Tables II and III), as judged from the label recovered from the internal organs. The amount of wax absorbed was in the same range as that of triolein, regardless of the basis of comparison, since the molecular weights of the 2 substances do not differ much (wax average MW—605; triolein—885). The absorbed label of both substances reached all the internal organs; contents in the skin and epididymal fats/g tissue exceeded those in other organs.

The content of wax absorbed did not increase with time because the content of label in the organs one day postinjection was similar to that on day 23 (Table II). Moreover, there was even a small decrease with time.

Most of the label was not absorved, as it was recovered from the carcass lipids even at 90 days after injection. Since the results varied greatly, no conclusion could be drawn about the fate of this label. Trials aimed at recovering the wax from the injection area by tissue digestion (unreported data) resulted in recovery of only a small portion of the wax—8 days after injection ca. 35% of the injected label were recovered from animals injected with 9 mg wax, 13% from the 25-mg-injected animals and 2% from the 120-mg-injected ones. Most of the lipid apparently diffused from the site at which it was injected. We would like to suggest that macrophages are responsible for the diffusion of the wax, since it is known that oils can induce macrophage formation (9). Indeed, in histophatological studies (Meshorer et al., unpublished data) an increase in macrophage population was observed in rats injected with jojoba wax.

Although most of ^{14}C in the carcass lipids remained as wax esters, there are indications that some of the absorbed wax was metabolized: (a) in TLC profile, label was found in spots other than those of wax esters, mainly in the triglycerides and free fatty acid location (Tables IV and V); (b) some label also was found in the aqueous fractions, mainly at 90 days after application (Table II).

In comparison with the wax, triolein was metabolized to larger extent, i.e., there was a higher level of label in the internal organs (Table II) as well as higher incorporation into polar lipids (Tables IV and V). The incorporation of triolein into body triglycerides could not be studied at this stage, as ^{14}C in the injected triolein could not be distinguished from ^{14}C in body triglycerides.

During the study, although large doses of the wax were injected to a large number of mice, their health apparently was unimpaired and no gross pathological signs were observed. Currently, toxic studies are being performed in guinea pigs and rats in order to ensure that the absorbed wax does not cause any systemic effects. Acute toxicity tests showed no negative effect (3), as already mentioned.

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Cholesterol and Triglyceride Distributions in an Adult Employee Population: The Pacific Northwest Bell Telephone Company Health Survey

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ABSTRACT

Plasma cholesterol and triglyceride are presented for 4503 adult employees of the Pacific Northwest Bell Telephone Company. Cross-sectional age and sex specific means and percentiles are shown. Females are classified by use or nonuse of exogenous sex hormones. Comparisons are examined among these groups, between blacks and whites, and among education and occupation categories. In these cross-sectional data, cholesterol and triglyceride generally increase with age and exhibit distinct differences by sex and by hormone use.

INTRODUCTION

Total plasma cholesterol levels, reflecting chiefly the concentration of low density lipoproteins (LDL) are a well-established index of cardiovascular risk (1,2). Triglycerides, too, are often elevated in patients with premature coronary disease (3-5), although the utility of triglyceride levels in assessing atherosclerotic risk remains a subject of debate (1,2). Much of the literature has dealt with specific topics of hyperlipidemia (5,6), hormone use and lipids (7,8), age and lipid relationships (9-11), and sex differences with respect to lipid levels (5,10-12). This paper reports age, sex, race, hormone use, education and occupation influences on lipids in a free-living adult population.

In 1972, a nationwide collaborative study was initiated by the Lipid Metabolism Branch of the National Heart, Lung and Blood Institute to determine the prevalence of different forms of hyperlipidemia and their correlates and sequelae, especially atherosclerosis, in epidemiologically-defined populations (12,13). To initiate a study of this magnitude and complexity, a network of 12 Lipid Research Clinics was established at university medical centers across the United States and in Canada (Toronto and Hamilton, Ontario). The Northwest Lipid Research Clinic in Seattle, Washington, was established as one of 3 clinics on the west coast of the United States.

The strength of this study is in its statistical design, use of a standardized protocol for laboratory determinations, physical measurements and questionnaire administration, and its basis in an epidemiologically well-defined population of adults. All procedures, laboratory, clinical and interviewing, were performed by specially trained and certified personnel (14-16). The data collected locally were subjected to both local and national edit processes and verification was made between the 2 processes.

METHODS

Population

Previous studies of the relationship between hyperlipidemia and coronary heart disease have suffered by a lack of reference lipid data from a nondiseased or more generally representative population. The employees of the Pacific Northwest Bell (PNB) Telephone Company in King County, Washington, were chosen to provide such data to examine the relationships among age, sex, race, occupation, education and sex steroid hormone use relative to plasma lipid levels. According to company lists, there were 6358 PNB employees working in King County in mid-1972. The potential study subjects were approached sequentially through their departments starting in July 1972 and continuing through May 1974. Eligibility for this study was determined by criteria of age and permanent job status in a work unit at the time of contact. There were 6052 eligible employees between the ages of 20 and 65; 5000 (83%) agreed to participate and were screened.

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Demographic characteristics of the study participants were compared to 1970 U.S. Census data for the surrounding community. The participants closely resembled residents of King County and the Seattle-Everett Standard Metropolitan Statistical Area (SMSA), although there were more blacks and fewer other nonwhites than in the local community (17).

The age, sex, race and occupation characteristics of both participants and nonparticipants were also analyzed to identify possible sources of bias in the results. Lower participation rates were found among females (81%), in the youngest (79%) and oldest (78%) age groups, among blacks (72%), and in the semiskilled job class (76%). The extremes of the occupation scale gave the best response (94%), although the lowest occupation group included only 16 employees. All categories had participation rates above 70%, and in most cases above 80%.

In order to assess the lipid distributions in a fasting, nonpregnant population, 38 pregnant women and 124 admittedly nonfasting individuals were excluded from analysis. Blacks below age 50 were sufficiently well represented to make some comparisons to whites (Table I); however, blacks as well as other nonwhites were excluded from the remainder of the tables and plots because of small numbers. This report, then, presents data for 4503 white, fasting, nonpregnant employees of Pacific Northwest Bell.

Data Collection

Data were gathered at the employee's individual work sites using a brief questionnaire administered by a trained interviewer. Sex hormone use among females was determined by asking: "Are you taking oral contraceptives, estrogens, or pills for hot flashes or to regulate periods?" The name or composition of the hormone medication was not asked. Each

TABLE I

Cholesterol and Triglyceride (mg/dl) Summary Statistics for Whites and Blacks[a] by Sex, Age and Hormone Status

	Cholesterol			Triglyceride	
Age/race	n	Mean	SD	Mean	SD
Males					
20-29					
White	436	176.	32.2	104.	67.4
Black	39	176.	42.1	81.	52.7
30-39					
White	599	192.	33.5	129.	113.1
Black	15	189.	35.5	106.	37.6
40-49					
White	655	208.	33.8	146.	144.7
Black	5	195.	18.1	76.	19.4
Female nonusers[b]					
20-29					
White	534	171.	29.1	67.	31.3
Black	55	177.	32.0	66.	41.1
30-39					
White	277	183.	32.7	77.	36.8
Black	24	191.	37.3	74.	41.9
40-49					
White	311	199.	34.6	97.	101.4
Black	6	186.	20.2	84.	49.7
Female users[b]					
20-29					
White	498	181.	30.5	100.	37.2
Black	60	172.	34.5	77.	29.4
30-39					
White	102	192.	31.8	110.	47.9
Black	14	177.	21.0	97.	33.9
40-49					
White	158	203.	35.6	119.	72.7
Black	3	176.	5.0	98.	20.4

[a]There were no blacks age 50-59; 1 black male and 1 female nonuser age 60-65 are not shown.

[b]Indicates use or nonuse of sex steroid hormones.

employee was asked his or her job title at PNB and the level of education attained. Job titles were categorized according to a modified Hollingshead scale (derived from *Two Factor Index of Social Position*, August B. Hollingshead, 1957, and "Occupational Classification System," U.S. Bureau of the Census, *1970 Census of Population Alphabetical Index of Industries and Occupations*). Education was classified into a 7-level scale ranging from "Less than 7 years of school" to "Graduate and professional training."

A blood sample for cholesterol and triglyceride analysis was collected from subjects who had fasted for at least 12 hr. Samples were extracted with zeolite and isopropanol and analyzed by Lipid Research Clinic Program continuous flow (AutoAnalyzer I) procedures (18). Cholesterol was determined by a ferric chloride color reagent (19) and triglycerides by a fluorometric 2,4-pentane dione procedure (20). There was a coefficient of variation of less than 4% and accuracy within 3% of target value for cholesterol analysis, and a coefficient of variation less than 5% and accuracy within 3% for glyceride analysis. Cholesterol target value concentrations were established by the Lipid Standardization Laboratory at the Center for Disease Control (CDC), Atlanta, on serum pool samples by the Abell-Kendall reference method (14,21). Plasma triglyceride target values were established by the CDC using a modified Carlson method (22).

Data Analysis

Summary statistics including the mean, standard deviation and percentiles (5, 10, 25, 50 = median, 75, 90, 95) describe the distributions of cholesterol and triglyceride for the 3 sex-hormone groups: males, females not using sex steroid hormones and females using such hormones. The general shape of the distributions is indicated by histograms and the relationships of lipids and age are described graphically. A one-way analysis of variance model was used to estimate age and sex-hormone differences within and among the 3 groups. The nonparametric Kruskal-Wallis analysis of variance procedure was run to verify the parametric analyses of variance test. Duncan's Multiple Range test (23) was used to identify specific age, sex and hormone differences in cholesterol and triglycerides. The 5% significance level was used for all statistical testing.

RESULTS

Cholesterol and triglyceride are examined with respect to age, sex, race, education and occupation. Data are sex, age and sex-hormone specific for adults ages 20-65.

Effects of Education

The 4503 white participants are distributed across 3 education groups: 54% have a high school education or less, 33% have some college education and 31% have at least a bachelors degree. The mean cholesterol and triglyceride values increase with decreasing education. The largest differences in mean lipids are between the 2 education extremes whereas the incomplete college education group has lipid values intermediate to the other 2 groups. Males with a college education have a mean ± SD cholesterol of 194 ± 37 mg/dl compared to a mean of 201 ± 36 mg/dl for males with a high school or less education. Mean triglycerides for these 2 groups of males are 115 ± 86 mg/dl and 132 ± 115 mg/dl. Female nonusers in the high and low education groups have average cholesterols of 182 ± 32 mg/dl and 191 ± 38 mg/dl, respectively, and mean triglycerides of 69 ± 29 mg/dl and 87 ± 72 mg/dl. College-educated female users have mean cholesterol and triglycerides of 189 ± 34 mg/dl and 98 ± 53 mg/dl. These values for users with a high school or less education are 197 ± 37 mg/dl and 117 ± 65 mg/dl. It should be noted that, on the average, the college-educated people are 3-6 years younger than those with no college education and the incomplete college education group has the same average age as college graduates.

Effects of Occupation

The Hollingshead codes for occupation were aggregated into 3 categories: 24% of the participants were classified into managerial, 43% into clerical-sales and 32% into craft positions. People holding managerial positions are 4-7 years older and have higher average cholesterol values than those in the craft positions. The average cholesterol for people in managerial positions is 201 ± 5 mg/dl compared to 190 ± 35 mg/dl for those in craft positions. Triglycerides show no association with occupation level. The highest mean triglycerides are in men classified in the clerical-sales category, 144 ± 133 mg/dl, whereas the lowest mean is in men in the highest occupation positions, 125 ± 103 mg/dl. The average ages for these 2 groups of men are 43 and 38 years, respectively. For females not taking hormones, those holding the managerial positions are the oldest, 40 years, but have the lowest triglyceride, 79 ± 42 mg/dl. Those in craft positions are the youngest, 33 years, but have the highest values, 87 ± 75 mg/dl. Females taking hormones and in management positions are older, 35 years, and have

TABLE II

Cholesterol (mg/dl) Summary Statistics and Percentile Distributions for Whites by Age, Sex and Hormone Status

Subjects	n	Mean	SD	Range	Percentiles						
					5	10	25	50	75	90	95
Males											
20-29	436	176.	32.2	96-306	131	138	154	171	193	219	232
30-39	599	192.	33.5	102-302	138	150	168	189	211	238	253
40-49	655	208.	33.8	120-327	156	168	185	206	227	251	272
50-59	499	211.	34.3	122-314	159	166	187	210	234	255	270
60-65	61	213.	33.9	157-307	163	171	187	214	234	248	285
Female nonusers[a]											
20-29	534	171.	29.1	97-292	126	136	151	168	186	212	225
30-39	277	183.	32.7	88-293	137	145	161	182	202	225	240
40-49	311	199.	34.6	120-334	147	156	174	197	220	242	261
50-59	162	220.	36.1	124-323	173	179	193	217	241	268	287
60-65	34	225.	28.1	163-296	182	190	205	334	346	258	272
Female users[a]											
20-29	498	181.	30.5	96-309	136	145	160	178	197	220	231
20-39	102	192.	31.8	107-282	148	155	168	190	211	234	244
40-49	158	203.	35.6	119-311	157	163	177	198	225	255	270
50-59	159	219.	32.3	134-294	164	176	195	218	242	260	267
60-65	18	247.	43.3	178-330	—[b]	192	207	238	268	304	—[b]

[a]Indicates use or nonuse of sex steroid hormones.

[b]Sample size too small to estimate percentile value.

the higher triglyceride values, 116 ± 66 mg/dl than the clerical-sales or crafts women ages 35 and 31, respectively, who have the same means, 109 ± 60 mg/dl.

Cholesterol Distributions

Comparing mean and median (50th percentile) cholesterol concentrations in Table II, the mean values are generally 2-4 mg/dl higher than the medians, reflecting a slight positive skewness, but not a striking departure from a normal distribution for any of the sex-hormone groups (Fig. 1).

Within each of the sex-hormone groups there is a significant increase in the mean cholesterol concentrations with age. Figure 2 shows that mean cholesterol rises steadily from 168 mg/dl in the youngest age group of males, to 212 mg/dl for men in their late 40s. Beyond this age the cholesterol means level off. Males in the 2 younger age decades are significantly different from each other as well as from the older age groups, which have similar values (Table II). For female nonusers, the cholesterol increases steadily between ages 20 and 39, rises sharply between ages 40 and 50, and then begins to level off. Mean cholesterol concentrations for female users do not show a leveling off at the older ages.

Cholesterol levels for young female nonusers are lower than for comparably aged men and female users. These trends change in the 50-65 age groups where the 2 female groups exhibit higher values than do males.

Statistical comparisons made within each age stratum across the 3 sex-hormone groups show that the 20-year-olds in all 3 groups have significantly different means from each other whereas 30-39-year-old female nonusers have significant mean differences from female users and males of the same age. As shown in Figure 2, a crossover effect is evident in ages 40-49 and 50-59. The 2 40-49-year-old female groups have similar and lower mean concentrations than do males. However, in the 50-59 age decade nonusers and users have similar and significantly higher mean concentrations than do males. The trend continues into the 60-65 age group.

Triglyceride Distributions

Mean triglyceride concentrations exceed the medians by 4-42 mg/dl (Table III), an effect more marked in the older age groups. Figure 3 shows that triglyceride has a highly peaked and positively skewed distribution. The highest triglyceride measurement in this population is 1728 mg/dl with 36 individuals having values greater than 500 mg/dl.

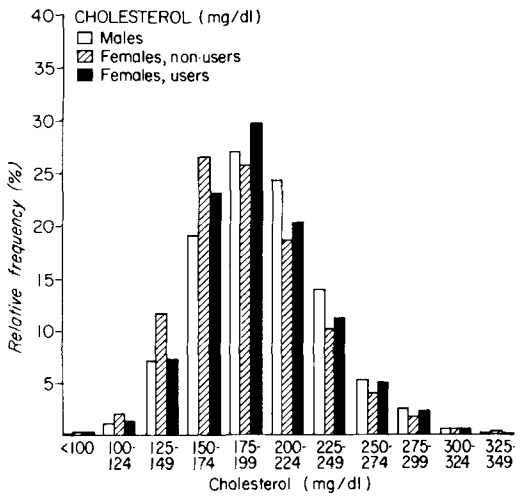

FIG. 1. Relative frequency distributions of cholesterol values for 4503 white participants by sex and hormone status: males, n = 2250; female nonhormone users, n = 1318; female hormone users, n = 935.

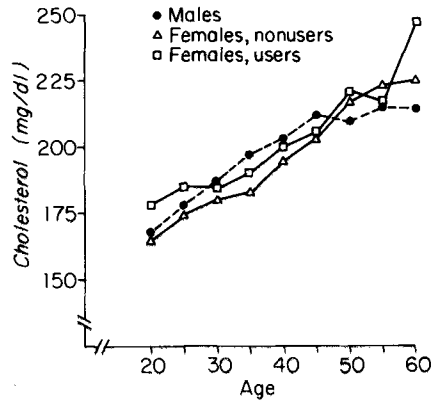

FIG. 2. Five-year mean cholesterol values for 4503 white participants vs age by sex and hormone status.

Within each of the 3 sex-hormone groups, mean triglyceride concentrations generally increase with age; the exceptions are 40-59-year-old males and 60-65-year-old female nonusers (Fig. 4). Males 20-29 years old have significantly lower triglyceride values than all other age groups which are statistically similar to each other (Table III). The 20-29- and 30-39-year-old female nonusers have significantly different mean triglycerides from each other and from the older age groups. For each age group of the female users, triglyceride means are significantly different from all nonadjacent age groups.

TABLE III

Triglyceride (mg/dl) Summary Statistics and Percentile Distributions for Whites by Age, Sex and Hormone Status

Subjects	n	Mean	SD	Range	Percentiles						
					5	10	25	50	75	90	95
Males											
20-29	436	104.	67.4	25- 800	42	48	62	86	119	176	239
30-39	599	129.	113.1	18-1404	51	58	75	100	145	211	276
40-49	655	146.	144.7	26-1728	55	64	82	113	165	225	300
50-59	499	139.	98.6	31-1336	54	63	85	115	167	231	270
60-65	61	153.	129.2	44- 704	64	67	88	117	170	234	407
Female nonusers[a]											
20-29	534	67.	31.3	17- 233	31	38	47	61	77	102	127
30-39	277	77.	36.8	25- 228	38	45	53	65	88	122	160
40-49	311	97.	101.4	32-1544	42	50	59	77	104	147	190
50-59	162	115.	100.0	37-1120	45	54	67	90	135	192	241
60-65	341	115.	54.2	55- 247	56	63	78	93	131	201	225
Female users[a]											
20-29	498	100.	37.2	20- 333	49	59	75	96	119	147	166
30-39	102	110.	47.9	38- 352	42	55	77	102	130	159	181
40-49	158	119.	72.7	38- 680	49	57	74	105	142	184	220
50-59	159	128.	82.4	40- 606	56	62	76	101	154	216	272
60-65	18	154.	105.1	64- 476	–[b]	64	74	112	176	235	–[b]

[a]Indicates use or nonuse of sex steroid hormones.

[b]Sample size too small to estimate percentile value.

Women not taking hormones have consistently and considerably lower values than comparably aged males and females taking hormones. Younger females taking hormones have triglyceride values similar to those for males. However, their 95th percentile values are considerably lower than those for males. Proportionately more males than females have triglyceride values in excess of 500 mg/dl: 1.4% of the males compared to 0.2% and 0.3%, respectively, of the female nonusers and users.

Statistical analysis confirms differences between the sex-hormone groups within age decades. Young female nonusers have significantly lower triglycerides than both female users and males who are similar. The 2 female groups ages 40-49 have comparable and each significantly lower triglycerides than males. In the 50-year-olds, the 2 female groups and female users and males are not statistically different; however, female nonusers and males are different from each other.

Racial Comparisons

Blacks are the predominant nonwhite race group, (n = 233) and are primarily 20-39-year-old females. Table I contains means and standard deviations of cholesterol and triglyceride for blacks and whites by sex-hormone group. Triglycerides are consistently lower for blacks across all age strata and for all 3 sex-hormone groups. Except for female nonusers ages 20-39, mean cholesterol values are lower in blacks than whites. Lipid levels generally show an increase with age for both blacks and whites.

DISCUSSION

This report presents plasma cholesterol and triglyceride data for an epidemiologically well-defined population of 4503 adult employees of the Pacific Northwest Bell (PNB) Telephone Company. While measurements of cholesterol and, to a lesser extent, triglycerides, have been obtained in various populations and reported over the past 30 years, no large population-base study in the Pacific Northwest has been presented. Determinations of inter-population and regional differences in blood lipids by Lewis et al. (24), Ricci et al. (25) and other researchers (26,27) set a precedent for having descriptions and distributions of lipids for varied ethnic groups and populations. Therefore, these data are of value in that they: (a) provide comparisons of plasma lipids between black and white racial groups, (b) depict recent data indicating differences between adult males, females taking and females not taking sex hormones, and (c) show the differential effects of sex-hormone treatment in younger and older women. Additionally, the relationships of cholesterol and triglycerides with education and occupation level are described.

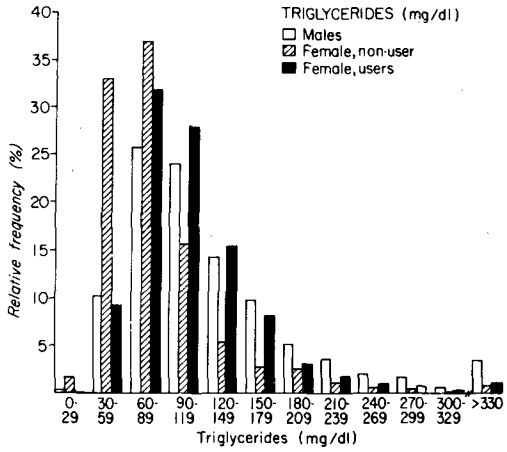

FIG. 3. Relative frequency distributions of triglyceride values for 4503 white participants by sex and hormone status.

Comparisons with Other Studies

The PNB men and women have mean cholesterol values similar to comparably aged men and women participants of the Toronto Lipid Research Clinic (28) which followed the same protocol and laboratory methods. However, for males in this study, triglycerides are on the average 20 mg/dl lower than those of Toronto males. With respect to both hormone groups, mean triglycerides for Toronto's

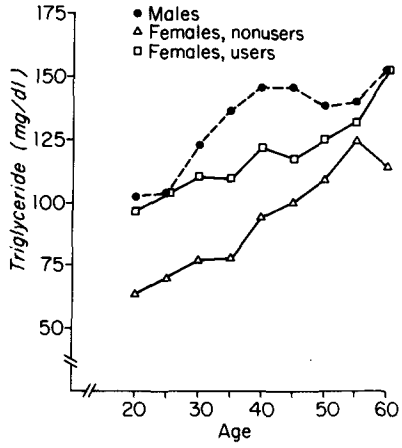

FIG. 4. Five-year mean triglyceride values for 4503 white participants vs age by sex and hormone status.

younger females, 20-49 years, are 10-20 mg/dl higher than the PNB females. The opposite is true for women ages 50-65.

Our values for male cholesterol are similar to those reported by Wood et al. (9) in a Central Valley, California, study. Plasma triglycerides were, on the average, lower among the PNB men. The California study did not classify women by hormone status, thus precise comparisons are not possible. However, with respect to cholesterol, their younger women are comparable to our hormone users whereas their older women have averages higher than either of the PNB female groups. Triglycerides for the younger California women are intermediate to our 2 hormone groups and comparable to our hormone users for ages 50-65.

Comparisons of our data to the 2 just described, as well as to other large population-base studies such as Framingham (29), HANES (30) and Tecumseh (31), show similarities and differences in some age-sex strata. Some of these studies, however, used plasma whereas others used serum; results from the LRC collaborative study (32) have shown determinations on plasma lipids to be ca. 3% lower than those done on serum when the same laboratory methodology is used. Lipid concentrations are influenced by diet, exercise, obesity and genetic factors, all of which could account for interpopulation or regional variations. These considerations strengthen the argument for comparing various data sets, rather than relying on results from a single study. Potential causes and ramifications of these differences should be considered before percentile or average plasma lipid values are applied epidemiologically or clinically.

Racial Differences

Blacks in the study population have lower triglyceride concentrations than whites. Cholesterol values on the average are also lower in blacks, with the exception of females between the ages of 20 and 39 and not using hormones. These findings agree with data from the collaborative LRC and the Evans County, Georgia, (33) studies. Given the information available, it is difficult to assess the relative importance of racial factors, but lipid differences between blacks and whites seem to be established.

Education and Occupation Influences

A slight but consistent trend of higher mean plasma lipids with lower education is seen for both males and females. Age, however, is also inversely related to education level in this population. Cholesterol is ca. 5% higher among people with a high school or less education. Triglyceride is ca. 20 mg/dl, 22% higher for the lowest education group than for the college graduates. Cholesterol is ca. 6% greater, and mean age 6 years older, for individuals in management than it is for those in skilled and unskilled craft jobs. Triglycerides do not show a consistent trend with occupation. The highest average triglyceride is seen for males classified as clerks or salesmen, female nonusers in craft positions and female users in management. In addition to age, other physiological and psychosocial factors may be reflected in these education and occupation differences.

Lipids and Age

These data are cross-sectional from adults ages 20-65 and cannot demonstrate that individuals' lipid concentrations rise with age. However, there is a positive association between lipid levels and age across this age range. This relationship for cholesterol is relatively strong in younger males and females not taking hormones, then weakens after about age 45. Females taking hormones show a constant increase through age 65. The association between triglyceride and age is most pronounced in younger males and older women. For males, this relationship is curvilinear, reaching a peak during the ages 40-49 and decreasing thereafter. This might result from selective survival, lifestyle or metabolic differences. Longitudinal, not cross-sectional, data are needed to address these issues.

Sex and Sex Hormone Effects

A particular strength of this study is the large number of women, 42%, who reportedly were on exogenous sex hormone therapy. This has allowed us to make not only comparisons between sexes, but to assess the impact of hormones on lipid levels in women. Assuming that menopause does not occur before the mid-40s, hormone therapy in young women usually indicates oral contraceptive use whereas older women take estrogen replacement hormones. Our results confirm previous studies which have found that premenopausal women have lower cholesterol concentrations than men and that a crossover in the mid-40s results in older women having higher levels than men (5,9,10,29). By contrast, triglycerides are lower in women compared to men at all ages between 20 and 65, the difference being most pronounced between males and nonhormone-taking females. Lower mean triglyceride levels in women compared to men have been seen in other population groups in North America

(5,9,10,29) and Scandinavia (34,35).

The conclusions are that nonhormone-taking premenopausal females have lower triglyceride and cholesterol concentrations than males. However, after age 45, male lipids no longer increase whereas postmenopausal female lipids continue to rise; cholesterol exceeds male levels and triglyceride approaches male levels. Women on hormone therapy at all ages have higher lipids than nonhormone-taking women, an effect more pronounced in the premenopausal years. Further analysis of the effects of specific hormones on lipoprotein fractions may point to hormone use as a risk factor for cardiovascular disease in women. At minimum, the risk of hyperlipidemia attributable to hormone use in younger women warrants attention and further investigation.

ACKNOWLEDGMENTS

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Partition of Ketone Bodies into Cholesterol and Fatty Acids in vivo in Different Brain Regions of Developing Rats

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ABSTRACT

The proportions of labeled ketone bodies and glucose incorporated into cholesterol and fatty acids in different regions of the brain in developing rats were compared. In cerebrums of 15- and 18-day-old rats, the ratios of dpm cholesterol/dpm fatty acids incorporated from [3-^{14}C] acetoacetate and [3-^{14}C] β-hydroxybutyrate ranged from 0.4 to 0.7, or 50 to 100% higher than values obtained with [U-^{14}C] glucose. Much higher ratios were obtained with younger animals: from 1 to 12 days of life, the values ranged from 1.0 to 1.3 with [3-^{14}C] β-hydroxybutyrate as substrate, and, from 1 to 5 days, with [3-^{14}C] acetoacetate, they were 1.0 or greater. During the first 12 days of life, the ratios resulting from administration of [U-^{14}C] glucose were 0.4-0.7. Clearly, a greater proportion of acetoacetate and β-hydroxybutyrate was incorporated into cholesterol during the first week of life than the remaining suckling period. Like cerebrum, other brain regions (i.e., cerebellum, midbrain, brain stem and thalamus) yielded higher ratios of dpm cholesterol/dpm fatty acids from [3-^{14}C] β-hydroxybutyrate during the first 12 days of life than on day 17. Brain stem was the most active region for lipid synthesis, and had the highest dpm cholesterol/dpm fatty acid ratio. Since active synthesis of cholesterol from ketone bodies during the early postnatal period coincides with a period of rapid brain growth, the results indicate that ketone bodies are more important early in the suckling period as sources of cholesterol for brain growth.

INTRODUCTION

Although the physiological significance of mild ketosis in newborns is not completely understood, ketone bodies (KB) are recognized as important precursors for the synthesis of brain lipids in vitro (1,2) and in vivo (3). Recently, Webber and Edmond (4) reported that in 18-day-old rats, the relative amount of β-hydroxybutyrate (β-OHB) or acetoacetate (AcAc) incorporated into sterols vs fatty acids were greater than that of glucose. It was demonstrated that for every 2 portions of KB used for fatty acid synthesis, one portion was used for cholesterogenesis. When glucose was used as a substrate, the ratio of fatty acids to cholesterol synthesized from the substrate was 4:1 (4). The observed difference in use of KB and glucose for synthesis of cholesterol was attributed to an active cytoplasmic pathway for production of acetoacetyl CoA (AcAc CoA) from AcAc. The generated AcAc CoA serves as a precursor for β-hydroxy-β-methyl glutaryl CoA and hence, cholesterol synthesis. Glucose, by contrast, must be converted to acetyl CoA (Ac CoA) via citrate synthase in mitochondria and ATP-citrate lyase in cytoplasm before entering the pathway for cholesterol synthesis in cytoplasm (5). It has been demonstrated in this laboratory that the activities of brain AcAc CoA synthetase increases after birth and remains at a maximal level during the second and third weeks of life (2). Since cholesterol content increases with increasing brain size and maturity (6), it is important to determine the developmental changes in cholesterol and fatty acid synthesis from ketone bodies in the brain throughout the postnatal period.

The results reported in this article indicate that KB are used more heavily for cholesterol synthesis immediately after birth and during the first week of life than during the remaining suckling period.

MATERIALS AND METHODS

Sprague-Dawley suckling rats were obtained in this laboratory by breeding procedures described earlier (7). They were suckled by their dams at all times. Male rats (200-250 g) fed Purina rat chow were used as a reference group. A single 10 μCi dose of [3-^{14}C] acetoacetate (30 mCi/mmol), DL-[3-^{14}C]-β-hydroxybutyrate (60 mCi/mmol) or [U-^{14}C] glucose (5 mCi/mmol) in saline was injected subcutaneously between the scapulae of each rat. In a preliminary study, it was found that the amount of ^{14}C-labeled, chloroform/methanol-extractable lipids recovered in the brain did not differ at 7, 24 and 72 hr after injection of the ^{14}C-labeled substrates. Therefore, in all experiments, animals were decapitated at 24 hr after injection of the labeled substrate. The brains

were immediately removed for separation of cerebrum, cerebellum, midbrain, brain stem (i.e., pons and myelencephalon) and thalamus (i.e., hypothalamus and thalamencephalon). Lipids were extracted according to the Folch et al. procedure (8). After saponification in 10 ml of 10% KOH in methanol, fatty acid and nonsaponifiable lipid fractions were extracted separately with three 5 ml-portions of petroleum ether (bp 50 to 60 C). The nonsaponifiable fraction was further precipitated with digitonin for isolation of cholesterol. The radioactivity associated with fatty acids and cholesterol was determined by methods described elsewhere (9).

[U-^{14}C]Glucose, ethyl [3-^{14}C]acetoacetate and DL-[3-^{14}C]-β-hydroxybutyrate were purchased from New England Nuclear, Boston, MA. Before each experiment, ethyl [3-^{14}C]-AcAc was converted to [3-^{14}C]AcAc by the Krebs and Eggleston method (10). Organic solvents used for lipid extraction were obtained from Fisher Scientific Co., Pittsburgh, PA. Digitonin and other chemicals were provided by Sigma Chemicals Co., St. Louis, MO.

RESULTS

The relative utilization of ^{14}C-labeled substrates for synthesis of lipids was determined by calculating ratios of dpm in cholesterol to dpm in fatty acids/g of wet cerebrum. The ratio resulting from AcAc administration was 1.4 on day 1, but decreased steadily during suckling to reach the adult level on day 1 (Fig. 1). The ratio observed after β-OHB administration increased from 1.0 on day 1 to a maximum of 1.3 on day 5. This maximal ratio was obtained until day 12, and then declined to the low level seen in adult brain. During the entire suckling period, excluding the 5th day, the ratios resulting from glucose administration were below 0.5 (range 0.32 to 0.47). In adult rats, the ratio obtained with glucose was 0.2.

The synthesis of cholesterol and fatty acids also was investigated in various brain regions of developing rats. For comparison of synthetic capacities in different brain regions from the same age group of animals, the activity was expressed as dpm of DL-[3-^{14}C]-β-OHB incorporated into lipids (cholesterol and fatty acids)/g of wet tissue. In 1-day-old rats, the activities did not differ among the 5 regions (Table I). During the period of 3-17 days, however, brain stem had the highest synthetic activity among all other regions. In 5- and 17-day-old rats, activities were lower in cerebrum and thalamus than in cerebellum and midbrain. The ratios of dpm in cholesterol to

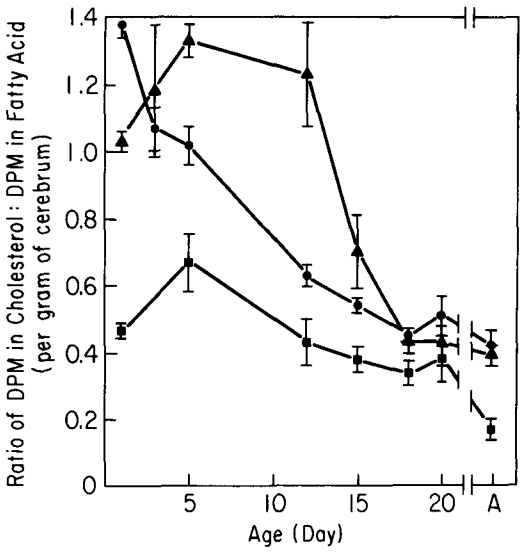

FIG. 1. Relative utilization of ketone bodies and glucose for cholesterol and fatty acids synthesis in cerebrum of developing rats. Ten μCi of each ^{14}C-labeled substrate was injected subcutaneously between scapulae of each rat. At 24 hr after the injection, rats were decapitated and cerebrum removed for determining radioactivity of ^{14}C-labeled substrate recovered in cholesterol and fatty acids. Ratios of dpm in cholesterol to dpm in fatty acids/g of wet cerebrum were determined from [3-^{14}C]acetoacetate (●—●), DL-[3-^{14}C]-β-hydroxybutyrate (▲—▲), and [U-^{14}C]glucose (■—■). Each point represents a mean ± SEM for 5 rats. The letter A on the abscissa denotes adult rats fed ad libitum.

dpm in fatty acids also were determined for different regions. In the cerebrum, the ratios were higher during the first 12 days of life than on day 17. In cerebellum and midbrain, the ratios were similar in 12-day-old and younger rats, but decreased on day 17. By contrast, the ratios in brain stem increased from 0.5 on day 1 to 2.9 on day 12 and started to decline on day 17. In thalamus, the ratio was low on day 1 (i.e., 0.6), but increased to 1.0-1.2 during the remaining suckling period. During the entire suckling period, except on day 1, brain stem had the highest ratio among all 5 regions.

DISCUSSION

Cholesterol and fatty acids are essential for cell proliferation in developing brain (6). Although differential use of KB and glucose for synthesis of cholesterol and fatty acids in whole brain of 15- and 18-day-old rats has been reported (4,11), their use during the earlier postnatal period has not been studied. In an earlier cross-labeling study, Webber and Edmond (4) demonstrated that ^{14}C-labeled AcAc,

β-OHB and glucose appeared in blood immediately after subcutaneous injection, reaching a maximal concentration in ca. 2 min for KB and 30 min for glucose, and decreasing exponentially thereafter. There was very little interconversion of KB and glucose as a function of injection times. These authors further suggested that the 3 ^{14}C-labeled substrates enter the brain solely in the form that was injected, although insignificant amounts of label could be converted to other metabolites before entering the brain for synthesis of fatty acids and cholesterol. [3-^{14}C]AcAc, DL-[3-^{14}C]-β-OHB and [U-^{14}C]glucose were therefore used to compare the relative use of KB and glucose for lipogenesis throughout the entire suckling period.

In agreement with previous reports (4,11), this study with cerebrum demonstrates that during the third week of suckling (i.e., 15 and 18 days), ratios of dpm cholesterol/dpm fatty acid synthesized from AcAc and β-OHB were 0.4-0.7 or 1.5- to 2.0-fold of values obtained with glucose. The ratios were much higher in younger animals. During the first 5 days, the ratios were 1.0 to 1.4 in rats injected with ^{14}C-labeled AcAc or β-OHB. Since active myelination occurs between 12 to 24 days after birth (12), these results indicate that more KB are incorporated into fatty acids than cholesterol during the active myelination, whereas before active myelination, at least equal amounts of either AcAc or β-OHB are used for cholesterol and fatty acid synthesis. In contrast to KB, glucose was predominantly used for fatty acid synthesis. Throughout the suckling period, cholesterol synthesis from glucose accounted for only 31 to 47% of fatty acid synthesis.

Cholesterol content in brain increases with the age of the animal and size of the brain (6). The rate of cholesterol deposition increases after birth, reaching a maximal value between 7 and 9 days and declining rapidly thereafter (13). It is apparent that the active synthesis of cholesterol from KB during the first week of life coincides with an increased accumulation of cholesterol in the brain. This, together with a rapid increase in brain weight (6,11), indicates the importance of KB as sources of cholesterol for brain growth early in the suckling period.

In these experiments, rats were injected with 10 μCi of ^{14}C-labeled AcAc, β-OHB or glucose, regardless of body weight and pool size of the substrates. The results, therefore, do not permit a comparison of the quantitative importance of the substrates for synthesis of cholesterol and fatty acids. During the suckling period, however, there are striking metabolic changes: (a) increased levels of KB but low levels of glucose in plasma (14); (b) increased uptake of KB from circulation by the brain (15); (c) increased activities of ketone-utilizing enzymes, i.e., 3-oxo-acid CoA transferase, AcAc CoA thiolase, β-hydroxybutyrate dehydroge-

TABLE I

Incorporation of DL-[3-^{14}C]-β-Hydroxybutyrate into Cholesterol and Fatty Acids in Brain Regions of Developing Rats[a]

Brain region[b]	Age (day) 1	3	5	12	17
	DPM × 10^{-3} recovered in lipids[c]/g tissue				
Cbr	265 ± 31[d]	198 ± 33	136 ± 13	162 ± 13	73 ± 7
Cbl	301 ± 26	250 ± 20	209 ± 22	264 ± 20	99 ± 9
Mb	231 ± 53	211 ± 15	189 ± 22	198 ± 18	110 ± 11
Bs	327 ± 35	356 ± 37	294 ± 15	378 ± 33	231 ± 26
Th	225 ± 33	253 ± 9	123 ± 9	134 ± 13	88 ± 13
	Ratio of DPM in cholesterol:DPM in fatty acids[c]				
Cbr	1.03 ± 0.03	1.17 ± 0.18	1.34 ± 0.05	1.23 ± 0.19	0.64 ± 0.11
Cbl	0.97 ± 0.01	0.95 ± 0.05	0.92 ± 0.05	1.13 ± 0.21	0.71 ± 0.08
Mb	1.01 ± 0.04	1.10 ± 0.24	1.00 ± 0.03	1.19 ± 0.19	0.64 ± 0.02
Bs	0.51 ± 0.14	1.55 ± 0.18	1.44 ± 0.04	2.86 ± 0.32	1.23 ± 0.17
Th	0.60 ± 0.13	1.18 ± 0.09	1.02 ± 0.04	1.02 ± 0.10	1.08 ± 0.13

[a]Ten μCi of DL-[3-^{14}C]-β-hydroxybutyrate was injected subcutaneously between the scapulae of each rat. At 24 hr after injection, rats were decapitated and brains removed for separation of different regions.

[b]Brain regions: Cbr, cerebrum; Cbl, cerebellum; Mb, midbrain; Bs, brain stem; Th, thalamus.

[c]Lipids represent a sum of cholesterol and fatty acids that were measured individually (Materials and Methods) for determining the ratio of dpm cholesterol/dpm fatty acids/g of wet brain tissue.

[d]Mean ± SEM for 5 rats.

nase and AcAc CoA synthetase (2,16-19); and (d) lower activities of glucose-utilizing enzymes such as hexokinase, pyruvate dehydrogenase and citrate synthase (20). Thus, it is reasonable to postulate that, during postnatal development, KB could become the major precursor for cholesterol and fatty acid synthesis in vivo. In fact, studies with cerebral slices (1) and whole brain homogenates (2) have shown that KB are preferred over glucose for lipid synthesis in the developing brain.

Why ketone bodies are more cholesterogenic than glucose is not completely understood. Webber and Edmond (4) postulated that a direct incorporation of AcAc CoA, derived from AcAc in cytoplasm, into β-hydroxy-β-methyl glutaryl CoA could facilitate cholesterol synthesis. This hypothesis is consistent with the previous finding that the activities of cytoplasmic AcAc CoA synthetase in developing brain increases after birth and is maintained throughout suckling at levels that are 2-fold higher than those in mature brain (2). An increase in activity of the synthetase could cause a rapid production of AcAc CoA in cytoplasm. Cholesterol synthesis from glucose, by contrast, involves citrate synthesis in mitochondria, transport and cleavage of citrate to AcCoA in cytoplasm (2). The generated AcCoA then enters cholesterogenic pathway by first converting to β-hydroxy-β-methyl glutaryl CoA. Besides the regulation of this pathway by ATP-citrate lyase, one must consider the enzymic regulation of citrate production. Since, as we have discussed, the developing brain has lower activities of citrate synthease, pyruvate dehydrogenase and hexokinase (20), the production of citrate from glucose might be limited. This, in turn, would not only lower fatty acid but cholesterol synthesis as well.

Finally, this study disclosed that, throughout the suckling period, brain stem was the most active site of lipid synthesis including cholesterol and fatty acids. Moreover, this region yielded the highest ratio of cholesterol/fatty acids after administration of β-OHB. Consistent with the contention that brain regions do not grow and develop at the same rate and at the same time (6,21), these results suggest that the type and quantity of lipids synthesized during development vary according to the requirements of different brain regions.

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Effect of Diet Composition and Fasting on Lipogenesis in Lean and Polygenic Obese Mice

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ABSTRACT

A line of mice was developed which exhibited spontaneous obesity when fed commercial laboratory ration low in fat content. Obese mice were compared to a nonobese related line to determine whether energy source in the diet would affect onset of obesity. Experimental diets–beef tallow (38% of calories as beef fat and 2% as corn oil), corn oil (40% corn oil) or low-fat (2% corn oil)–were instituted ad libitum at the time of weaning. When the mice reached 6 months of age, lipogenesis was investigated by injecting intravenously ^3H_2O and glucose-U-^{14}C. ^3H_2O and glucose-U-^{14}C incorporation into fatty acids of fed mice was greater for obese than for lean mice. Fatty acid synthesis was inhibited by high-fat diets compared to low-fat diet in both lines. Of the 2 high-fat diets, the corn oil diet inhibited fatty acid synthesis about twice as much as beef tallow diet. There was no line effect on tritium incorporation into cholesterol. Cholesterol synthesis from glucose-U-^{14}C was greater in obese than lean mice. Diets had no effect on tritium and glucose-U-^{14}C incorporation into cholesterol. Fasting reduced fatty acid synthesis in all mice, but total body fatty acid synthesis was not affected by lines or dietary treatment under fasted conditions. These data suggest that degree of lipogenesis, in part, explains obesity. A failure of inhibition of lipogenesis or an enhanced efficiency in fat deposition by feeding beef tallow compared to corn oil diet may explain the fact that lean mice fed the beef tallow diet tended to be more obese that lean mice fed corn oil or low-fat diets.

INTRODUCTION

The homozygous recessive genotype, ob/ob in the mouse and fa/fa in the rat, exhibit hyperphagia and excessive lipid deposition (1-4). Similar findings have been reported for polygenic obese mice (5,6).

Numerous indirect methods have been employed to study lipogenesis in vitro and in vivo in obese rodents by using labeled carbon precursors (7-12) or by studying lipogenic enzyme activities (see review by Romsos and Leveille, 13). Total lipogenesis estimated by the use of tritiated water has not been investigated as frequently in obese rodents (14,15).

There is general agreement that a lipogenic state is produced by high-carbohydrate diets and that high-fat diets depress lipogenesis (13). Polyunsaturated fats have been shown to decrease fatty acid synthesis to a greater degree than do saturated fats (16-19). Fasting has been found to inhibit lipogenesis (20,21).

This study was designed to investigate genetic control vs dietary regulation of total body fatty acid and cholesterol synthesis using a polygenic obese animal model.

MATERIALS AND METHODS

Four lines of mice were developed in a long term (over 50 generations) selection experiment. The constitution of the lines was reported by Sutherland et al. (6). The polygenic obese male mice used in our study were descendants of a cross between lines 1 and 3 necessitated by low fertility. The lean male mice were descendants of line 4.

Animals were randomly assigned to diets at weaning. Composition of the diets is similar to the ones fed to rats by Dupont et al. (16,22) and is shown in Table I. The beef tallow diet provided a polyunsaturated-to-saturated (P/S) ratio of ca. 0.2, whereas the corn oil diet provided a P/S ratio of ca. 5.

All mice were scheduled for metabolic study and necropsy at 6 months of age. Mice were fed either ad libitum or fasted for 24 hr. A tracer mixture of 956×10^2 dpm of glucose-U-^{14}C and 122×10^5 dpm of ^3H_2O (New England Nuclear, Boston, MA) per g body weight was injected intravenously into each mouse. Specific activity of the glucose used was 4.20 mCi/mmol. Each animal was placed in an adjustable mouse restrainer and the exposed tail was heated with a heat lamp (Sylvania 50 watt reflector track light) to dilate the vein immediately before tracer injection. All mice were killed 180 min after injection by immersion into liquid nitrogen. A homogeneous mixture of total carcass was obtained by grinding each frozen animal in a homogenizer (Tekmar Company, Cincinnati, OH) using liquid nitrogen and Dry Ice. Homogenates of each mouse were stored in plastic petri dishes at -16 C until

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TABLE I

Composition of Diets

Ingredient	Low fat	Beef tallow	Corn oil
Lactalbumin[a]	15.0	19.0	19.0
Corn oil[b]	1.0	1.2	21.2
Beef tallow[c]	–	20.0	–
Salt mix[a,d]	4.0	5.0	5.0
Cellulose[a]	2.0	2.5	2.5
Cornstarch[e]	39.9	25.3	25.3
Sucrose[f]	36.5	25.0	25.0
Vitamin mix[g]	1.6	2.0	2.0

[a]General Biochemicals, Inc., Chagrin Falls, OH.

[b]Mazola, obtained from local retail market.

[c]Fat rendered from beef kidney knobs obtained from the Department of Animal Sciences.

[d]Teklad, Madison, WI. Composition reported in ref. 23. Chromium at 70 ppb and selenium at 30 ppb were added.

[e]Amaizo, American Maize Products Co., New York.

[f]Great Western United, Denver, CO.

[g]Contained per 2 kg of vitamin mixture made up with cornstarch: vitamin A acetate, 0.8 g (500,000 IU/g); vitamin D (calciferol), 0.5 g (400,000 IU/g); α-tocopherol acetate, 40.0 g (500 IU/g); menadione, 0.1 g; vitamin B_{12}, 10.0 g; biotin, 0.05 g; folic acid, 0.2 g; *p*-aminobenzoic acid, 20.0 g; inositol, 20.0 g; Ca-pantothenate, 2.0 g; nicotinic acid, 3.0 g; pyridoxine-HCl, 1.0 g; riboflavin, 1.0 g; thiamin-HCl, 0.5 g; choline chloride 400.0 g; ascorbic acid, 10.0 g.

analysis.

One g of homogenate of each animal was used for lipid extractions. Total lipids were extracted as described by Folch et al. (24). In this study, 30 ml of chloroform/methanol (2:1) to 1 g of tissue was used. Lipids were saponified and nonsaponifiable lipids were extracted with petroleum ether. After acidification with HCl, fatty acids were extracted with petroleum ether. A toluene-based cocktail containing 4 g of Omnifluor (New England Nuclear, Boston, MA) per liter of toluene was used and radioactivity was determined in a liquid scintillation counter, a Nuclear-Chicago Unilux II system set for dual label counting and equipped with external standardization. The counter was on-line with a Hewlett Packard computer which was programmed to correct for background, quenching and to calculate dpm of ^3H and ^{14}C. The incorporation of tritium into total body fatty acids and cholesterol is expressed as μmol of acetyl groups (25,26) incorporated into the lipids/g of mouse at 180 min after injection. The specific activity of labeled water remained constant from the time of injection until animals were sacrificed. Data were analyzed by 3-way analysis of variance (27).

RESULTS

Pooling diet and regimen, live weights of lean and obese mice (Table II) were significantly ($P < 0.001$) different at 6 months of age. Obese mice fed corn oil or low-fat diets weighed more than their lean counterparts; however, the obese mice fed beef tallow were not heavier than the lean mice fed the same diet (Table II).

Pooling diet and regimen, fatty acid synthesis as determined from ^3H_2O and glucose-U-^{14}C incorporation was significantly ($P < 0.05$) greater in the obese mice compared to their lean counterparts (Figs. 1 and 2). Fatty acid synthesis, measured either by ^3H_2O or by glucose-U-^{14}C, appeared to be inhibited by the corn oil and the beef tallow diets relative to the low-fat diet in both the lean and obese mice (Figs. 1 and 2). In the fed, lean mice, fatty acid synthesis from glucose was inhibited 26% by the beef tallow diet and 66% by the corn oil diet compared to the low-fat diet. For obese mice, these values were 27 and 60%, respectively. Generally, in fed mice, both ^3H_2O and glucose-U-^{14}C incorporation into fatty acids was greatest in mice fed the low-fat diet,

TABLE II

Live Weights of Lean and Obese Mice at Six Months of Age

Genotype, diet	Mice fed ad libitum	Mice fasted 24-hr
Lean, beef tallow	45.06 ± 10.21[a]	41.46 ± 2.78
Obese, beef tallow	48.92 ± 8.52	49.44 ± 7.15
Lean, corn oil	40.58 ± 6.88	40.24 ± 4.98
Obese, corn oil	49.98 ± 10.95	49.54 ± 6.56
Lean, low fat	37.34 ± 5.48	28.90 ± 2.86
Obese, low fat	48.38 ± 7.94	46.10 ± 8.55
LSD[b]	9.2	

[a]Mean ± SD (n = 5).

[b]Least significant difference at 5% level (27).

intermediate in the mice fed beef tallow and least in the mice fed corn oil; fasting obliterated these differences. All mice fed ad libitum incorporated significantly ($P < 0.001$) more tritium and glucose-U-^{14}C into fatty acids than 24-hr fasted mice.

Genetic line effect on cholesterogenesis, determined from ^3H_2O, was nonsignificant (Fig. 3). However, obese mice incorporated significantly ($P < 0.05$) more glucose carbon into cholesterol compared to their lean counterparts (Fig. 4). Cholesterol synthesis, as measured by ^3H_2O and glucose-U-^{14}C, was unaffected by diet. As with the fatty acid synthesis, mice fed ad libitum synthesized more cholesterol than 24-hr fasted mice. In contrast to its effects on fatty acid synthesis, fasting apparently did not obliterate line and diet effects on cholesterogenesis.

DISCUSSION

Pooling diets and regimen produced polygenic obese mice that were ca. 25% heavier and had greater percentage of whole body fat (28) than the lean mice at 6 months of age. The lean and obese mice fed the beef tallow diet weighed the same, whereas the obese mice fed the corn oil and low-fat diets were heavier than their lean counterparts at 6 months of age. Percentage of fat in obese mice fed the beef tallow diet was similar to the fat content in their lean counterparts which was not observed in the mice fed corn oil and low-fat diets (28). Thus, diet overcame genetic line effect in the obese mice fed beef tallow. Calorie intake was higher in obese mice fed both beef tallow and corn oil compared to their lean counterparts, whereas food intake in lean and obese mice fed the low-fat diet was not different (unpublished data). Thus, not just a positive calorie imbalance but probably a difference in utilization of energy for maintenance or growth must account for why lean and obese mice fed beef tallow weighed the same and obese mice fed corn oil or low-fat diets were heavier than their lean counterparts at 6 months of age.

In this study, ^3H_2O incorporation into fatty acids of fed mice was greater for the obese than for the lean mice. This finding agreed with that of Hems et al. (14) who measured incorporation of ^3H_2O into liver and adipose tissue fatty acids of ob/ob obese mice. In contrast, Jansen et al. (29) who administered oral doses of glucose-U-^{14}C to hyperglycemic-obese mice, observed an increase in labeled hepatic, but not extrahepatic, fatty acids in obese compared to nonobese mice. Hyerpglycemia and insulin resistance in these animals may indicate decreased glucose uptake by extrahepatic tissues (30). Cawthorne and Cornish (15) measured

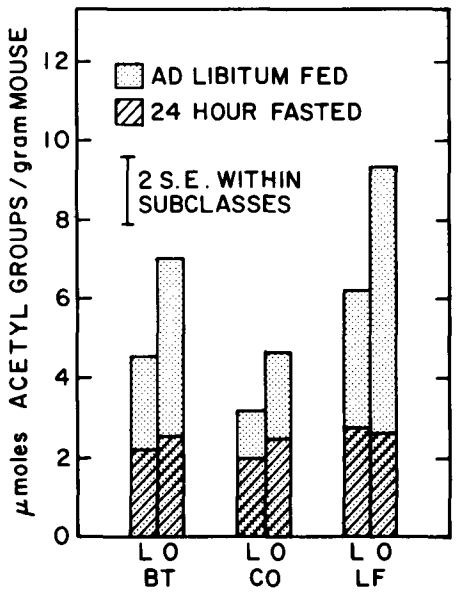

FIG. 1. Effects of diet composition and fasting on incorporation of tritiated water into total body fatty acids at 180 min after injection. Each variable (strain, diet and regimen) represents the average of 5 mice. BT: beef tallow; CO: corn oil; LF: low fat; L: lean; O: obese.

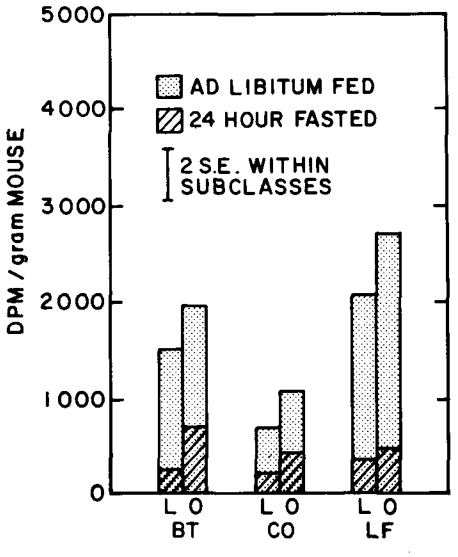

FIG. 2. Effects of diet composition and fasting on incorporation of glucose-U-^{14}C into total body fatty acids at 180 min after injection. Each variable (strain, diet and regimen) represents the average of 5 mice. BT: beef tallow; CO: corn oil; LF: low fat; L: lean; O: obese.

fatty acid synthesis in lean and ob/ob obese mice 60 min after interperitoneal injection of ^3H_2O. A high-corn-oil diet (410 g corn oil/kg diet) compared to a low-fat diet (35 g corn oil/kg diet) decreased fatty acid synthesis in adipose tissue and in the "rest of carcass" but not in the liver of lean mice. In ob/ob obese mice, however, fatty acid synthesis in adipose tissue and "rest of carcass" was unaffected, but liver fatty acid synthesis was reduced, suggesting hyperinsulinemia in ob/ob obese mice (15). By summing responses of the different tissues to approximate total body response, high-corn-oil feeding decreased fatty acid synthesis by 51% in lean and 37% in ob/ob obese mice compared to low-fat diet. Similar results were obtained with our mice. The effect of high-corn-oil feeding on lipogenesis in individual organs, however, needs to be determined in our polygenic model to draw more direct comparisons with the ob/ob obese mice. Data do not distinguish between de novo synthesis and fatty acid chain elongation, and the assumption was made that the major event is de novo synthesis, not chain elongation. That point requires further clarification.

Fasting the animals for 24 hr reduced fatty acid biosynthesis in both lines. The lean and obese mice, whether fed beef tallow, corn oil, or low-fat diets, responded in the same degree to fasting. Of the 2 high-fat diets, corn oil inhibited fatty acid synthesis in both lines about twice as much as beef tallow relative to low-fat. In the lean, fed mice, the corn oil diet inhibited fatty acid synthesis from glucose 66% compared to the low-fat diet. Similar results were obtained by Jansen et al. (31), who administered glucose-U-^{14}C orally to mice, then killed the animals 60 min later. In both the lean and obese mice fed corn oil ad libitum, more labeled glucose apparently was used for other metabolites than for de novo synthesis of fatty acids relative to mice fed beef tallow or low-fat diets. These results indicate that our polygenic obese mice are able to respond metabolically to diets differing in kind and amount of fat. Regulation of the response, however, seems to contribute to obesity.

Much less (ca. 80% less) labeled water and glucose were incorporated into total body cholesterol than into total body fatty acids. Glucose-U-^{14}C incorporation into cholesterol was greater in all polygenic obese mice compared to their lean counterparts, suggesting a sparing of carbon metabolites other than glucose in the obese animals. Although non-significant, both high-fat diets appeared to inhibit cholesterogenesis compared to the low-fat diet, which was in contrast with Du-

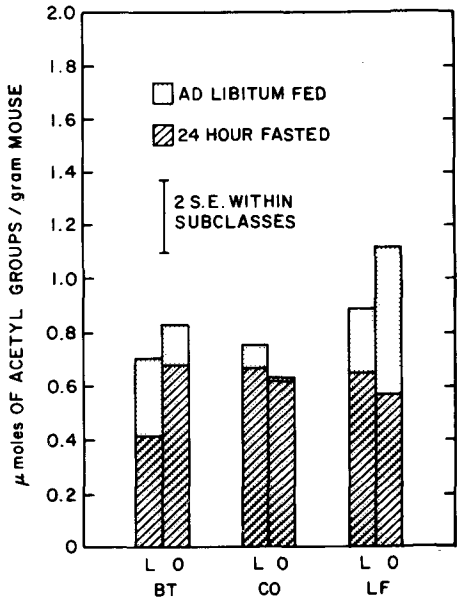

FIG. 3. Effects of diet composition and fasting on incorporation of tritiated water into total body cholesterol at 180 min after injection. Each variable (strain, diet and regimen) represents the average of 5 mice. BT: beef tallow; CO: corn oil; LF: low fat; L; lean; O: obese.

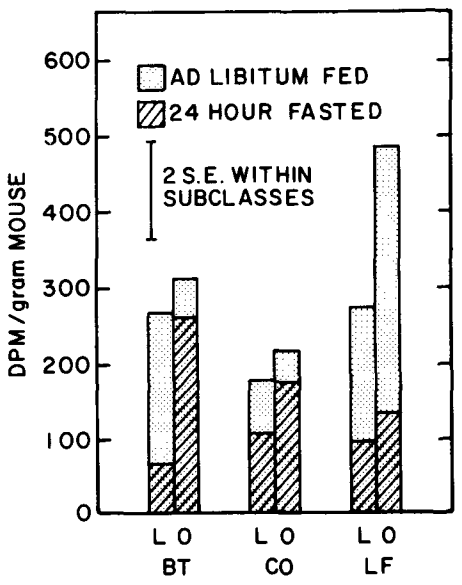

FIG. 4. Effects of diet composition and fasting on incorporation of glucose-U-^{14}C into total body cholesterol at 180 min after injection. Each variable (strain, diet and regimen) represents the average of 5 mice. BT: beef tallow; CO: corn oil; LF: low fat; L: lean; O: obese.

pont's early work (16,22), where [^{14}C]acetate was used as a substrate. Glucose compared to acetate can be considered to follow a more physiological pathway toward the synthesis of cholesterol. Glucose must first enter the mitochondrial acetyl-CoA pool, whereas acetate can directly enter the extra-mitochondrial acetyl-CoA pool. Fasting inhibited cholesterogenesis in all lean and obese mice.

In summary, there apparently is a strain and diet interaction in lipogenesis in polygenic obese mice. These interactions suggest that dietary management, as well as genetic predisposition, may lead to obesity.

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Hormonal Regulation of Medium Chain Fatty Acid Synthesis by Mouse Mammary Gland Explants

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ABSTRACT

Explants of pregnant mouse mammary tissue were cultured in media supplemented with various hormones. During the last few hours of culture, explants were labeled with [^{14}C] acetate. Fatty acid synthesis by the tissue was analyzed using reverse phase thin layer chromatography, and incorporation of radioactivity into the medium chain fatty acid (MCFA) fraction was calculated as total MCFA per mg tissue fresh weight and as a percentage of the total fatty acid radioactivity (%MCFA). After 48 hr of culture, explants had an elevated %MCFA synthesis only when exposed to media containing insulin, cortisol and prolactin, confirming previous reports. Increasing doses of prolactin (maximal response at 300 mg/ml) caused a coordinate rise in both %MCFA synthesis and total MCFA synthesis per mg fresh weight. Epithelial cells isolated from explants after culture for 48 hr with insulin, cortisol and prolactin showed an elevated %MCFA synthesis compared to cells from explants cultured with insulin and cortisol alone, indicating that hormonal stimulation of mammary explants causes a change in the chain length of fatty acids synthesized by mammary epithelial cells. The specificity of the explant response to prolactin was tested with other hormones: FSH and calcitonin had no effect, whereas bovine growth hormone showed activity only at high concentrations. Progesterone and 17β-estradiol also had no effect. The analysis of MCFA synthesis provides another means of (a) assessing hormonal action upon mammary tissue, and (b) evaluating the biological activity of prolactin.

INTRODUCTION

In vitro organ culture has been frequently used to investigate the response of mammary tissue to hormones (1,2). In such studies, tissue responses have been assessed using both histological and biochemical criteria. Among the biochemical criteria is the analysis of milk-specific mammary gland products, such as casein (3,4) or α-lactalbumin (5,6). However, the role of hormones in mammary gland differentiation is incompletely understood. The analysis of additional biochemical characteristics might give further insight into this process.

Milk contains a high concentration of triglycerides, and the synthesis of fatty acids for milk fat is a major metabolic activity of lactating mammary tissue (7). In many species, a high proportion of the fatty acids in milk fat are of short or medium chain length, the exact composition varying between species. Lactating mice produce milk which has ca. 35% medium chain fatty acids (MCFA), of which lauric acid is the predominant form (7). The synthesis of MCFA represents a biochemical function unique to the mammary epithelial cell and appears to involve the synthesis of a chain-length specific fatty acylthioesterase (8,9).

The hormonal regulation of fatty acid synthesis in mammary tissue has been studied in several species, including the rabbit (10), rat (11) and mouse (12,13). In these studies, mammary tissue explants have been exposed to various hormone combinations during organ culture. The incorporation of [^{14}C] acetate into either total or specific fatty acids was determined after various incubation periods. From such work, it is apparent that the synthesis of both total and milk-specific fatty acids is hormonally controlled in a manner similar to other milk-specific products. In the rabbit, where mammary alveolar structures can be removed relatively free of adipose elements, the evidence suggests that the epithelial cells respond to such hormone treatment by modifying the chain length of fatty acids synthesized. However, in the mouse and the rat, the large number of adipocytes in the explants complicates the interpretation of such a response. In these cases, the synthesis of milk-specific fatty acids by explants after hormonal stimulation could be the result of a change in the spectrum of fatty acids synthesized by the alveolar cells and/or an increase in the synthesis of fatty acids by these cells.

In view of the importance of the mouse mammary gland system in studies of mammary gland development, we felt that the basis for the hormonal stimulation of MCFA synthesis should be clarified. However, traditional methodologies of lipid analysis (such as gas liquid chromatography) are time-consuming and insensitive, increasing the difficulty of determining MCFA synthesis. As a consequence,

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previous studies of mammary gland fatty acid synthesis usually have monitored hormonal effects on total fatty acid synthesis, restricting the analysis of specific fatty acid synthesis to a few, selected conditions. In this paper, we describe a method using reverse-phase thin layer chromatography (rp-TLC) to separate methyl esters of fatty acids from mammary tissue. The incorporation of radioactive precursor into medium and long chain fatty acid fractions can be quickly and easily determined for all experimental conditions. Using this procedure, the hormonal stimulation of mouse mammary explants during organ culture was investigated, and changes in fatty acid synthesis by the epithelial cells as a result of this stimulation were determined.

MATERIALS AND METHODS

Organ culture techniques used were basically as described by Talamantes (14). Mammary glands were removed from time-bred multiparous BALB/cCrgl mice and placed in sterile Waymouth's MB 752/1 culture medium (Gibco) containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). In some cases, the mammary fat pads had been previously "cleared" of epithelial elements (15); the absence of epithelial cells in these samples was verified by histological examination of several tissue pieces. Extraneous tissue was removed and the gland divided into small explants (0.5-1.0 mg each). Explants were washed with culture medium, and nonfloating tissue discarded. Five to 7 explants were transferred to hormone-supplemented culture media in multi-well tissue culture plates (Falcon 3008) and allowed to float freely. Tissue was incubated at 37 C in a humidified atmosphere of prefiltered O_2/CO_2 (95:5) for various lengths of time.

The following hormones were used: porcine insulin (Lilly Research Laboratory: 25.4 U/mg); ovine prolactin (NIH-P-S-10); ovine FSH (NIH-FSH-S-6); bovine growth hormone (NIH-GH-B-8); porcine calcitonin (Armour Pharmaceutical K330200B: 52 U/mg); cortisol (Calbiochem); 17β-estradiol and progesterone (Aldrich). Hormones were solubilized and then diluted with Waymouth culture medium to an appropriate concentration and the solutions were filter sterilized (Millipore, 0.4μ pore size).

In some experiments, mammary explants were first cultured in vitro and the epithelial cells then isolated by dissociation with collagenase (Worthington, type 3, 125 U/mg suspended in phosphate-buffered saline with 4% bovine serum albumin–10 mg enzyme/10 ml saline/gm tissue). The epithelial cells were filtered through a 50μ Nitex filter (Tetko Inc.), pelleted by low speed centrifugation, and resuspended in Waymouth medium. An aliquot of the suspension was analyzed for viability (typically greater than 95%) using trypan blue.

[^{14}C] Sodium acetate (Schwartz/Mann, 57 mCi/mmol) was added to give a final concentration of 5 μCi/ml, and at the end of the labeling period, the explants or cells were washed with cold Waymouth medium; explants were then weighed. Fatty acids were extracted and transesterified with 1 N methanolic HCl by heating for 2 hr at 70 C. After cooling, hexane and water were added, the sample was shaken, and the phases were allowed to separate.

For rp-TLC (16), Polygram Sil G sheets (Brinkman) were impregnated with a 7% solution (in hexane) of silicone oil (Dow Corning 1107 or Dow Corning 200; 20 c.s.). Fatty acid methyl ester standards (methyl decanoate, laurate, myristate and palmitate) and an aliquot of the hexane epiphase from a sample were applied to the origin. Chromatograms were developed (acetonitrile/acetic acid/water, 6:1:2, v/v), sprayed with a 1% solution of α-cyclodextrin (Eastman Chemical) in 60% ethanol, and visualized with iodine vapor. After dividing the chromatograms between the methyl laurate and myristate bands, the 2 sections were analyzed in a liquid scintillation counter (Packard Tricarb) using Omnifluor scintillation fluid (New England Nuclear). Radioactivity incorporated into the medium and long chain fatty acid fractions was determined; total fatty acid incorporation is the sum of these 2 fractions. Data are expressed as either incorporation/mg fresh weight of the sample, or as the percentage of the total fatty acid incorporation found in medium chain fatty acids (%MCFA). When either [^{14}C] palmitic or [^{14}C] lauric acid (New England Nuclear, 10 mCi/mmol) was added to unlabeled tissue samples, tranesterified, and separated by rp-TLC, over 90% of each was recovered in the correct fatty acid fraction.

Fatty acid methyl esters of some samples were collected after separation on a 4-ft 1% SE-30 column using a Varian 1200 gas chromatograph equipped with a flame ionization detector and a 1:10 effluent splitter (operating conditions: injector 220 C; detector: 250 C; oven: 120 C for 4-min followed by 200 C for 4 min). Using these conditions, medium chain fatty acid methyl esters (including methyl decanoate and laurate) eluted during the first 4 min; longer fatty acid methyl esters (methyl myristate through stearate) eluted during the subsequent 4-min period. The effluent was collected for each period in a glass U-shaped

tube immersed in an acetone/Dry Ice bath. After collection, the contents were washed directly into a counting vial with scintillation fluid. Collection efficiency was ca. 55% as determined with [^{14}C] methyl laurate and [^{14}C] methyl palmitate.

RESULTS

Several tissue samples were cultured with radioactive [^{14}C] acetate for 2 hr, analyzed by both rp-TLC and gas liquid chromatography (GLC), and the %MCFA of each sample calculated. Analysis of samples by either method yielded similar results. For example, mammary tissue from virgin, 13-day pregnant and 5-day lactating mice gave %MCFA values of 1.5, 10.7 and 58.9, respectively, when analyzed by rp-TLC, and 0.5, 10.6 and 53.8, respectively, when analyzed by GLC.

Using freshly dissected explants of mammary tissue from mice in the middle of pregnancy, several aspects of the in vitro labeling procedure were studied. Incorporation of [^{14}C] acetate into both MCFA and total fatty acids increased linearly with both increasing labeling periods from 1 to 3 hr and with increasing tissue mass. %MCFA of the explants, however, was not affected. Since tissue response expressed as %MCFA is minimally affected by such variables, most of the following data are calculated in this manner.

The role of mammary adipocytes in MCFA synthesis was investigated by labeling freshly dissected explants from mammary fat pads cleared of epithelial cells with [^{14}C] acetate for 2 hr. The %MCFA synthesis of fat pad tissue from virgin, 13-day pregnant and 5-day lactating mice was 1.3 (± SD = .1), 1.6 (.2) and 1.9 (.1), respectively, indicating that adipocytes are not responsible for the increase in MCFA synthesis seen in mammary tissue during pregnancy and lactation. This suggests that MCFA synthesis is primarily the role of the epithelial cells. That epithelial cells do synthesize MCFA is confirmed by studies using isolated epithelial elements (see following).

The ability of various hormones to regulate mammary fatty acid synthesis was determined using organ culture techniques. Mammary explants were incubated in culture media supplemented with various combinations of hormones for 48 hr. During the last 2 hr of culture, [^{14}C] acetate was added to determine the synthesis of MCFA (Table I). Only in the presence of insulin, cortisol and a lactogen was there a rise in %MCFA. The addition of sex steroids (17β-estradiol and progesterone) had no effect upon this tissue response. Tissue response to prolactin is dose-dependent; increasing doses of ovine prolactin caused a rise in both %MCFA synthesis and total MCFA synthesis in explants from mice in the middle of pregnancy (Fig. 1). A maximal response was

TABLE I

Synthesis of MCFA by Mammary Explants[a] Cultured with Various Hormones

Hormone treatment	MCFA[b] (%)
Insulin (I)[c]	2.9 (.1)
Cortisol (F)[c]	2.2 (.5)
Ovine prolactin (PRL)[c]	2.7 (.2)
I + F	2.7 (.5)
I + PRL	4.4 (.1)
I + F + PRL	12.0 (2.3)
I + F + PRL + 17β-estradiol (E2)[c]	10.8 (2.3)
I + F + PRL + progesterone (P)[c]	10.3 (1.1)
I + F + PRL + E2 + P	10.4 (2.3)

[a]Explants from a 15-day pregnant mouse were cultured for 48 hr in media supplemented with the indicated hormones. During the last 2 hr tissue was labeled with [^{14}C]-acetate.

[b]MCFA (%): percent of total fatty acid incorporation in the medium chain fatty acid fraction. Results represent the mean (± SD, n=3) in a representative experiment.

[c]Concentration of hormones: I=5 μg/ml; F=1 μg/ml; PRL=2 μg/ml; E2=10 ng/ml; P=1 μg/ml.

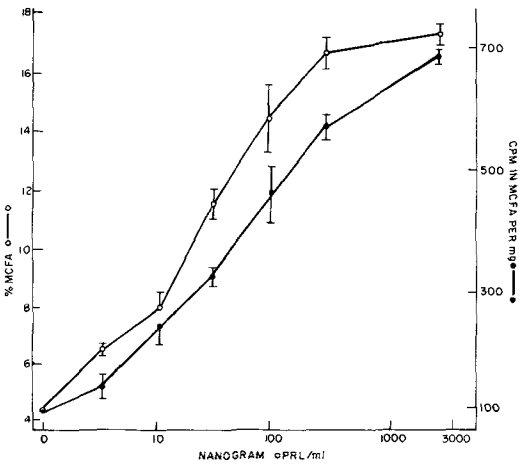

FIG. 1. Dose response of MCFA synthesis to ovine prolactin. Mammary explants from a 16-day pregnant mouse were cultured in media containing insulin (5 μg/ml), cortisol (1 μg/ml), and increasing amounts of ovine prolactin for 48 hr. During the last 2 hr, tissue was labeled with [^{14}C] acetate. Both the %MCFA (open symbols) and the total CPM incorporated into MCFA per mg tissue fresh weight were determined. Results indicate the mean and range of 2 observations in a representative experiment.

seen with doses of 300 ng or more of prolactin. The response of the explants in this culture system was maximal after 48 hr; explants cultured for 72 hr showed a decreased response to hormones.

The specificity of this response for prolactin also was determined. Explants were incubated in culture media containing insulin and cortisol and one of several other peptide hormones. As seen in Table II, a rise in %MCFA requires the presence of prolactin; FSH and calcitonin have no effect. Bovine growth hormone causes a small response (ca. 1% that of ovine prolactin).

The role of the epithelial cell in MCFA synthesis was further investigated by isolating epithelial cells from explants which had been cultured in hormonally supplemented media for 48 hr. As indicated in Fig. 2, epithelial cells isolated from explants show a higher %MCFA synthesis than intact explants, since the explants contain adipocytes synthesizing long chain fatty acids. However, there is a similar increase in %MCFA synthesis in intact explants and epithelial cells from explants cultured in medium containing insulin, cortisol and prolactin when compared to the %MCFA synthesis in explants or cells which had been cultured in medium containing only insulin and cortisol. This indicates that in vitro exposure to this 3-hormone combination can change the spectrum of fatty acids synthesized by the mammary epithelial cell.

TABLE II

Effect of Protein Hormones on MCFA Synthesis in Mammary Gland Explants[a]

Hormone (weight/ml)	MCFA[b] (%)
None[a]	4.5 (.1)
Ovine prolactin	
(4 ng)	6.5 (.1)
(33 ng)	11.4 (.7)
(300 ng)	16.6 (1.0)
Bovine growth hormone	
(100 ng)	7.5 (1.1)
(3000 ng)	11.3 (1.6)
Ovine FSH	
(600 ng)	6.1 (.2)
(5000 ng)	6.4 (.1)
Calcitonin	
(600 ng)	5.9 (.2)
(5000 ng)	6.6 (.2)

[a]Explants from a 17-day pregnant mouse were cultured for 48 hr in media supplemented with insulin and cortisol (5 μg and 1 μg/ml, respectively) and other indicated hormones. During the last 2 hr, tissue was labeled with [^{14}C]acetate.

[b]Results represent the mean (± SD; n=3) in a representative experiment.

DISCUSSION

The analysis of many mammary gland responses to hormones is complicated by the heterogeneous cellular composition of this tissue. This certainly is true of fatty acid synthesis, since it is not specific to a single cell type in the gland. However, MCFA synthesis apparently is a biochemical function specific to the mammary epithelial cell, as indicated by the analysis of cleared mammary fat pads and isolated epithelial cells (Fig. 2). These data are consistent with those of other authors (7).

The hormones necessary for stimulation of MCFA synthesis by mammary explants from pregnant mice during organ culture was determined. Under the conditions employed, insulin, cortisol and prolactin must all be present, confirming previous observations (12,13). The response is sensitive and specific for the presence of prolactin; both total and percentage synthesis of MCFA rise with increasing doses of ovine prolactin, with a maximal response at 300 ng/ml (Fig. 1). Thus, the rise in total fatty acid synthesis seen in explants cultured under similar conditions (12) appears to be the result

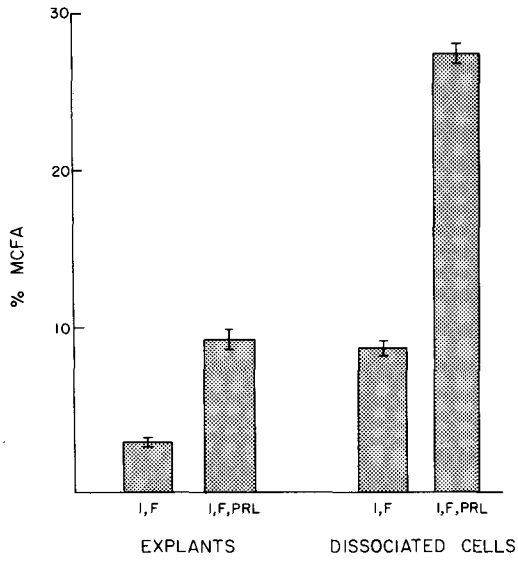

FIG. 2. Synthesis of MCFA by mammary explants and isolated epithelial cells. Mammary explants from a 17-day pregnant mouse were cultured in media containing insulin and cortisol (I,F; 5 and 1 μg/ml, respectively) or insulin, cortisol and ovine prolactin (I,F,PRL; 5, 1, and 2 μg/ml, respectively) for 48 hr. Intact explants or epithelial cells isolated from explants by collagenase treatment were labeled with [^{14}C]acetate for 2 hr and then analyzed for MCFA synthesis. Results represent the mean (± SD, n=3) in a representative experiment.

of prolactin regulation of MCFA synthesis at these doses, rather than the regulation of some additional aspect of explant lipid synthesis.

Of the other peptide hormones tested in this paper, only high concentrations of bovine growth hormones increased MCFA synthesis (Table II), consistent with its low activity in other bioassay systems (2); this may represent minor amounts of contaminating prolactin in this preparation. Other peptide hormones such as FSH and calcitonin, which do not belong to the prolactin/growth hormone family, have no effect on this system. The addition of an estrogen or progestin either alone or together had no effect upon MCFA synthesis. These data are similar to other biochemical responses of the mouse mammary gland to hormonal stimuli (5-7). One exception, however, is the reported inhibition of α-lactalbumin activity in mammary gland explants exposed to progesterone in addition to insulin, cortisol and prolactin (6). The lack of such an inhibition of %MCFA synthesis suggests differences in the hormonal control of these 2 mammary gland functions.

Because of the large adipocyte population in mammary tissue, there are several interpretations of an increase in MCFA synthesis by explants after in vitro hormonal stimulation. For example, an increase in MCFA synthesis could be the result of changes in total fatty acid synthesis by epithelial cells (due to an increase in cell number or metabolic activity). A change in total fatty acid synthesis by the epithelial cells would be detected as an increase in both total MCFA synthesis and %MCFA synthesis of the explant, even though the chain length of the fatty acids synthesized by the epithelial cells had not changed. Alternately, an increase in MCFA synthesis might reflect a change in the proportion of different fatty acids synthesized by the epithelial cells. As shown in Figure 2, explants and epithelial elements isolated from explants show a similar increase in %MCFA synthesis after cultivation with prolactin. This indicates that the hormonal effects observed during organ culture reflect, at least partially, changes in the proportion of different fatty acids synthesized by the epithelial cells. Thus, regulation of MCFA synthesis in mouse mammary tissue apparently is similar to that reported for the rabbit (10).

We are continuing to study prolactin/growth hormone regulation of mammary tissue MCFA synthesis. Because of its sensitivity and specificity, this characteristic also has proven useful for evaluating the biological activity of prolactin derivatives. It has proven especially useful for evaluating iodinated preparations, since the assay products (^{14}C-labeled lipids) can be easily separated from the iodinated material.

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The Influence of Linoleic Acid Intake on Membrane-bound Respiratory Activities

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ABSTRACT

The fatty acid composition of subcellular membranes, like that of depot fats, can be altered by dietary manipulation. Most attention has been directed toward the effects of feeding an essential-fatty-acid-free diet. We chose to examine some responses generated by the feeding of a dietary fat containing a disproportionately high level of an essential fatty acid. Rats were fed diets formulated with beef tallow (BT) to provide 4% (P/S, 0.2) or safflower oil (SO) to provide 24% (P/S, 7.6) of total energy as linoleic acid. Lipids isolated from hepatic mitochondria of rats fed the SO diet contained, in relative terms, 85% more unsaturated bonds. Mitochondria isolated from livers of rats fed either diet were tightly coupled. When all aspects of oxidative metabolism examined in this report are considered, mitochondria of SO group origin exhibited greater oxidative activities but lower ADP/O ratios than did BT mitochondria. Our hypothesis is that the perturbed state of the membrane-bound phospholipids initiates a remodeling-response through which an intramitochondria source of ADP is generated to support state-3 respiratory activity.

INTRODUCTION

The proportions of amino acids in the diet of an organism ordinarily deviate from the proportions in which they are required by the organism. Ingestion of a diet containing disproportionate amounts of amino acids will not commonly result in adverse effects; adverse effects from the ingestion of such a diet occur only when homeostatic mechanisms are deficient, defective or artificially overloaded (1).

The homeostatic mechanism of lipid metabolism apparently is more limited in scope (2); overloading may be accomplished within the limits of the food supply. The adaptive responses to a low-fat diet include increases in the hepatic capacity to synthesize saturated and monoenoic fatty acids (3); the latter capacity is elevated when the diet is deficient in linoleic acid (4). Ingestion of linoleic acid effectively reduces the hepatic capacities to synthesize saturated and monoenoic fatty acids (5,6). The influence of dietary components upon the composition of depot fat of animals has been known for generations (2). More recently, the influence of dietary fat components on the composition of hepatic subcellular lipids was demonstrated (6). The response to dietary fat composition is magnified in the fatty acid composition of hepatic mitochondrial phospholipids (7).

Occasionally, reports of the influence of dietary fat on specific metabolic parameters provide data regarding the overall performance of the experimental animal. In some, (8-12), but not all (13) reports, the weight gain and feed efficiency of rats (8,9), pigs (10), ruminants (11) and fish (12) were higher in groups fed the more saturated fat, providing the essential fatty acid requirements were satisfied.

In 1972, we reported that a diet providing 35% energy as safflower oil (SO), in comparison to a similar beef tallow (BT) diet, significantly increased the cytochrome aa_3 concentration and cytochrome c oxidase activity in avian hepatic mitochondria (14). The activities of succinic dehydrogenase and mitochondrial phosphoenolpyruvate carboxykinase were elevated in the hens fed the beef tallow diet.

The safflower oil diet supported lower weight gains. These observations suggested that the efficiency of oxidative activity was reduced subsequent to structural perturbations of the mitochondrial membrane introduced by the altered fatty acid composition of its constituent lipids. Initial studies of the effect of dietary lipids on activities in avian hepatic mitochondria were not pursued because of our failure to isolate these mitochondria in satisfactory respiratory control. In the following report, we describe the effects of these diets on energy metabolism in rats.

MATERIALS AND METHODS

Male Sprague-Dawley rats (210.5 ± 3.0 g) obtained from ARS-Sprague Dawley (Madison, WI) were housed individually in mesh-bottomed cages at the Animal Care Unit of the University of Wisconsin Medical School. Palatable diets with the capacity to bind up to 20% oil were formulated from the ingredients shown in Table

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TABLE I

Composition of Experimental Diets

Ingredient	Fat supplement	
	Beef tallow	Safflower oil
Ground yellow corn	495	470
Wheat middlings (standard)	75	100
Alfalfa meal	50	50
Soybean meal	225	225
Salt-vitamin mix[a,b]	55	55
Fat supplement[c]	100	100
Metabolizable energy (Kcal/g)	3.1	3.1
Protein (weight %)	16.4	16.6
Total fat (g/kg)	120	119
Total vitamin E[d,e]	45.1	89.8
Linoleic acid (g /kg)	14.6	86.6
α-Tocopherol:linoleic acid (mg/g)[f]	3.1	1.0
Sources of energy (%)		
Protein	20	20
Carbohydrate	45	45
Fat	35	35

[a]Salt-vitamin mix: dicalcium phosphate, 10 g; iodized salt, 5 g; calcium carbonate, 40 g; manganese oxide, 0.12 g; vitamin A, 10,000 IU/g, 2.20 g; vitamin D_3, 8800 IU/g, 0.84 g; vitamin E, 44 IU/g, 0.3 g; riboflavin, 0.55 g; vitamin B_{12}, 0.44 g; and Santaquin[b], 0.11 g. [b]Registered trademark of the Monsanto Chemical Company (6-ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline). [c]Each fat supplement contained 0.03% vitamin E acetate which was a gift of Hoffman-LaRoche Inc., Nutley, NJ. [d]Yellow corn, number 2,22 IU/kg; wheat middlings, standard, 21.3 IU/kg; alfalfa meal, 17% protein, 124.3 IU/kg (source, see ref. 15); safflower oil, 89 mg; total tocopherol (51.5% α tocopherol) 100 g (source, see ref. 17). [e]The requirement for vitamin E in diets containing up to 5% polyunsaturated acid is 30 mg/kg diet (18). [f]The requirement for vitamin E increases when the intake of polyunsaturated fatty acid increases but not with a linear relationship. The ratio calculated for the satisfactory human diet is 0.43; for rats, 0.2 when the diet contains 5% polyunsaturated fatty acid (19,20).

I. The 16.4% protein diet employed for this experiment provided 20, 35 and 45% of energy intake as protein, fat and carbohydrate, respectively at an energy density of 3.1 Kcal/g. Edible beef tallow (BT) (a gift from Oscar Mayer and Company, Madison, WI) or safflower oil (SO) (purchased from Pacific Vegetable Oil International Inc., Richmond, CA), was added to the diet; the diets as formulated provided 4 or 24% of energy as linoleic acid. The diets and water were given ad libitum for a period of 3-4 weeks. The rats were killed by decapitation, the livers were immediately removed, washed in a chilled medium of 40 mM Tris-HCl, pH 7.4, 250 mM sucrose and 1 mM EGTA and then homogenized in 8 vol of medium with a Potter Elvehjem-type homogenizer.

Mitochondria were prepared by differential centrifugation according to the Schneider method (21) modified by the use of the medium just described. Except when required by assay conditions, the mitochondria were held at ice temperature.

Methyl esters of the fatty acids in the extracts of the mitochondrial lipids (22) and the dietary fats were prepared by the Metcalf et al. method (23). Gas liquid chromatographic analyses of the fatty acid methyl esters were done on a 0.32 × 183 cm glass U-column packed with 6% diethylene glycolsuccinate polyester on Chromosorb W (60-80 mesh). The instrument, equipped with a hydrogen flame ionization detector, was operated isothermally at 165 C. The methyl esters were identified by comparing their retention times with those of standards. Quantitation was done by triangulation.

The oxygen consumption by isolated mitochondria was measured polarographically with a Clarke oxygen electrode as described by Estabrook (24). An aliquot of mitochondria (2 mg protein) was added to the oxygen buffer consisting of 20 mM KCl, 225 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 20 mM triethanolamine HCl, pH 7.4. State-4 respiration was determined following the addition of 6 μmol succinate and state-3 respiration following the addition of 0.6 μmol ADP. The total vol was 1.0 ml, the oxygen concentration at the time of the succinate addition was 0.24 mM and the temperature was maintained at 26 C. Ratios of respiratory control and [ADP]:[O]

were calculated from these tracings of oxygen consumption.

Cytochrome oxidase activity in frozen and thawed mitochondrial preparations was measured as described by Wharton and Tzagoloff (25). Mitochondrial succinate dehydrogenase, total and endogenous ATPase, adenine nucleotide translocase and [$^{32}P_i$]ATP exchange activities were determined by the respective methods of Nachbaur et al. (26); Lardy and Wellman (27); Klingenberg and Pfaff (28) and Boyer et al. (29). Mitochondrial phospholipase A activity was measured following the Waite and vanDeenen method (30), except that liberated fatty acids were determined photometrically (31). Inorganic phosphorous, ADP and ATP concentrations were determined according to the methods of Sumner (32), Lamprecht and Trautschold (33) and Adams (34). Mitochondrial protein was determined by the biuret reaction as adapted by Gornall et al. (35) with bovine serum albumin serving as the standard.

For a companion trial, male Sprague-Dawley rats (127.9 ± 5.5 g) were fed only between the "dark hours," 1700-0600, so that daily food intakes could be recorded. Livers and hearts were excised and weighed following slaughter. Thio barbituric acid numbers were determined as a measure of lipid peroxidation (36).

The basic components of the diet were purchased from the Poultry Research Laboratory, University of Wisconsin. The acyl CoA thioesters and [$^{32}P_i$] were purchased from P and L Biochemicals, Milwaukee, WI, and New England Nuclear, Boston, MA.

The remaining materials required for the analyses, lactate dehydrogenase, pyruvate kinase, glucose-6-phosphate dehydrogenase, hexokinase, cytochrome c, phenazine methosulfate, succinate, phosphoenolpyruvate, adenosine diphosphate, adenosine triphosphate, nicotinamide adenine dinucleotide (reduced) nicotinamide adenine dinucleotide phosphate (oxidized) and 2-thiobarbituric acid were supplied by Sigma Chemical Company, St. Louis, MO, and 1,1,3,3-tetraethoxypropane by Eastman Organic Chemicals, Rochester, NY.

RESULTS AND DISCUSSION

Fatty acid patterns of lipids extracted from rat hepatic mitochondria reflect the fatty acid pattern of the fat in the diet (Table II). When BT containing 3.4% linoleic acid was fed, the mitochondrial lipids contained 9.3% linoleic acid and 8.1% arachidonic acid. Linoleic and arachidonic acids were increased to 46.4 and 16.1% when SO, 76.3% in linoleic acid, was fed (Table II). The ratio of arachidonic acid to linoleic acid in mitochondrial lipids was about 1 when BT was fed; the ratio decreased to 0.35 when SO was in the ration. In relative terms, the lipids extracted from the hepatic mitochondria of rats fed the SO diet contained 85% more unsaturation than did the lipids extracted from livers of rats fed the BT diet.

The phospholipids, the major portion of mitochondrial lipids, exhibit the preferential incorporation of unsaturated fatty acids at the 2-position and saturated fatty acids at the 1-position. Thus, it was of interest to determine whether the perturbation in fatty acid composition of the membrane lipids by the highly unsaturated dietary fat might affect the activity of phospholipase A. This activity appeared to be increased by ca. 20% (NS) when the SO diet was fed (Table III). In related studies, phospholipase A activities in hepatic mitochondria of chicks (n=5) and guinea pigs (n=3) were increased 58% (8.4 ± 1.2 and 13.3 ± 1.6, nmol FFA/mg protein/min $P < 0.01$) and 24% (14.6 ± 1.2 and 18.1 ± 1.0 nmol FFA/mg protein/min, $P < 0.05$), respectively, when SO replaced BT in the diet.

Representative polarographic recordings of succinate-supported oxygen consumption by hepatic mitochondria from the 2 groups of rats appear in Figure 1. The respiratory control ratio calculated for rats fed the BT diet was

TABLE II

Fatty Acid Patterns Determined by Gas Liquid Chromatography

	Fat source		Mitochondrial lipids	
	Beef tallow	Safflower oil	Beef tallow	Safflower oil
Palmitic acid	26.9	7.1	22.8 ± 0.05	14.1 ± 0.9
Palmitoleic acid	4.9	–	5.2 ± 0.2	–
Stearic acid	23.1	3.1	16.5 ± 0.3	12.1 ± 1.1
Oleic acid	41.8	14.4	37.5 ± 0.3	11.3 ± 0.3
Linoleic acid	3.4	76.3	9.3 ± 0.5	46.4 ± 1.1
Arachidonic acid			8.1 ± 0.5	16.1 ± 0.8

TABLE III

Comparisons of the Influences of BT and SO Diets on Mitochondrial Enzyme Activities and Substrate Concentrations

	n	Units	Diet fat		t
			BT	SO	
Activity					
Phospholipase A	3	nmol FFA/mg prot/min	17.4 ± 1.2	21.0 ± 3.2	1.05
Respiratory control	6	State 3/state 4	5.44 ± 0.56	4.39 ± 0.47	1.44
Succinic dehydrogenase	6	nmol O_2/mg prot/min	52.15 ± 6.30	65.17 ± 7.07	1.37
Cytochrome c oxidase	6	nmol cyto. c/mg prot/min	755 ± 37	970 ± 63	2.94[b]
ADP/O	6	nmol ADP/nmol O_2	1.38 ± 0.03	1.21 ± 0.06	2.53[a]
ATP-[^{32}P]exchange	6	[^{32}P]mg prot/min	9000 ± 225	8325 ± 200	2.26[a]
ATPase					
Endogenous	6	nmol P/mg prot/min	29.7 ± 6.2	31.1 ± 5.6	0.23
Total	6	nmol P/mg prot/min	428 ± 35	472 ± 29	0.97
Substrates					
ADP	6	nmol/mg prot	1.70 ± 0.001	1.82 ± 0.020	6.00[d]
ATP	6	nmol/mg prot	4.89 ± 0.28	3.63 ± 0.06	3.71[c]
ATP/ADP	6		3.05 ± 0.17	1.95 ± 0.01	6.47[c]

[a] $P < 0.05$.
[b] $P < 0.025$.
[c] $P < 0.01$.
[d] $P < 0.001$.

5.44; that of rats fed the SO diet was 4.39 (chicks (BT), 2.60 ± 0.20; chicks (SO), 2.00 ± 0.25; guinea pigs (BT) 8.27 ± 0.73; guinea pigs (SO), 5.75 ± 0.75 ($P < 0.01$)). Although the ratios are not significantly different, the values are consistent with greater state-4 respiratory activity in mitochondria of the SO diet group (Fig. 1). The rate of oxygen consumption during succinate-supported state-3 respiration was ca. 25% (NS) greater in mitochondria of the SO-diet group (Table III). Mitochondria from guinea pigs fed the SO diet exhibited rates of oxygen uptake 55% ($P < 0.05$) greater than those from animals fed the BT diet (29.2 ± 3.6 and 45.4 ± 2.6 nmol O_2/mg protein/min). When succinate oxidation was coupled with PMS rather than the electron transport chain, mitochondria from chicks fed the BT-diet exhibited an activity 36% ($P < 0.05$) higher than that present in chicks fed the SO diet (4.00 ± 0.53 and 2.95 ± 0.38 A units/mg protein/min). These results suggest that the effect of the dietary fat is exerted at a point between the enzyme and the terminal acceptor. Cytochrome c oxidase activity was 28% ($P < 0.025$) higher in mitochondria from rats (Table III) and 55% ($P < 0.01$) higher in mitochondria from chicks fed the SO diet (10.60 ± 14.04 and 1640 ± 120 nmol reduced cytochrome oxidized/mg protein/min. The ADP/O ratio was 14% higher ($P < 0.05$) in rats fed the BT diet (Table III). Adenine nucleotide translocase activity was slightly higher (8%, $P < 0.05$) in the mitochondrial membranes isolated from rats fed the BT diet (Table III). The translocation of adenine nucleotides across the inner mitochondrial membrane constitutes a key role in energy-linked respiration. Because of this central role, any natural inhibitor could serve as a potential physiological regulator of mitochondrial oxidative metabolism. Fatty acyl CoA thioesters compete with adenine nucleotides for binding sites on the adenine nucleotide translocator (37). The degree of inhibition exerted by a specific long chain fatty acyl CoA thioester is a function of its chain length and degree of unsaturation (38). Adenine nucleotide translocase activity was inhibited 50% by 5 μM lauroyl CoA, 83% by 5 μM myristoyl CoA, 92% by 5 μM palmitoyl CoA, 85% by 5 μM stearoyl CoA and 36% by 5 μM oleoyl CoA. The dietary fat had no influence on these activities. The influence of the diet appeared when linoleoyl CoA was tested. This thioester was more inhibitory for the activity in mitochondria from the SO group. For these mitochondria, the activity was inhibited 5 ± 1.0, 9 ± 2.6 and 70 ± 5.4% by 5, 10 and 60 μM linoleoyl CoA. On the other hand, the activity in BT mitochondria was not inhibited (+3 ± 0.8 and +2 ± 0.5%) by 5 and 10 μM linoleoyl CoA and at 60 μM, the inhibition was 47 ± 4.0%. Each value represents 9 assays of the ATP-[^{32}P]-exchange reaction. At physiological concentrations of the acyl CoA thioesters, the adenine nucleotide translocase activities of BT and SO mitochondria were similarly inhibited. These data and the observation that total translocase activity was slightly higher (8%) in BT mitochondria indicate that the membrane of the SO mitochondria remained intact. Shown in Table IV are results obtained following the companion trial during which daily diet intakes were recorded. These data indicate that efficiency (g weight gain/g diet consumed) was marginally greater in the rats fed the BT diet. Whether or not the SO reduced metabolic efficiency cannot be assessed since body compositions were not analyzed. The BT diet promoted greater carcass growth, the result of either fat accumulation or lean tissue growth (or both).

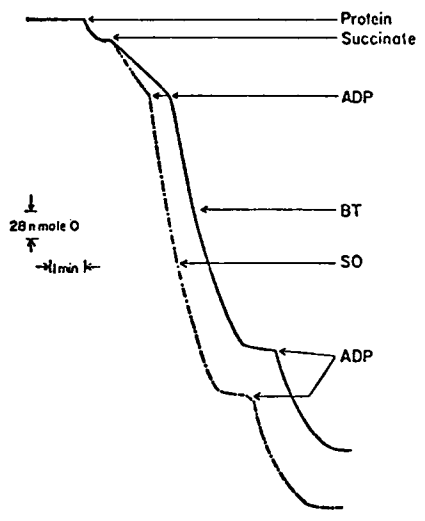

FIG. 1. Representative polarographic recordings of succinate-supported oxygen consumption by hepatic mitochondria from rats fed diets containing safflower oil (SO) or beef tallow (BT). Experimental details are given in Materials and Methods.

Respiratory functions of cardiac mitochondria are influenced by dietary erucic and elaidic acids (39-41). Diets rich in erucic acids alter mitochondrial structural-functional organization to the extent that energetic efficiency is impaired. Linoleic acid, unlike erucic acid, is a necessary component of membrane phospholipids. A major consideration when a diet high in linoleic acid is fed is the possibility that responses attributed to the accumulation of

TABLE IV

Weight Gain, Diet Consumption, Organ Weight and TBA Values of Rats Fed BT or SO Diets for 30 Days

	Diet		
	BT	SO	
n	5	5	t
Diet consumed (g)	516 ± 45.3	510 ± 45.0	0.09
Weight gain (g)	135.1 ± 9.23	121.0 ± 4.77	1.45
Organ weight (g)			
Liver	7.03 ± 0.870	7.60 ± 0.544	0.55
Heart	0.83 ± 0.054	0.94 ± 0.077	0.64
Organ (% body weight)			
Liver	2.674 ± 0.067	3.041 ± 0.125	2.76[b]
Heart	0.316 ± 0.002	0.378 ± 0.009	6.74[c]
Thiobarbituric acid number[a]			
Liver	4.01 ± 0.85	3.83 ± 0.33	0.20
Heart	1.90 ± 0.76	0.54 ± 0.13	1.77

[a]Mg 1,1,3,3-tetraethoxypropane/kg tissue.
[b]$P < 0.025$.
[c]$P < 0.001$.

linoleic acid in the membrane are, in reality, responses to a functional vitamin E deficiency. The SO diet provided 89.8 mg vitamin E/kg, a quantity 3-fold the requirements established for the rat. The ratio of α-tocopherol to linoleic acid in this diet was 1, a value suggestive of at least a 2-fold safety factor. Additionally, an antioxidant, Santaquin, was added to the diet. The TBA number for livers and hearts of rats from each diet group appear in Table IV. These data indicate that the level of antioxidants in the SO diet was sufficient to prevent peroxidative damage.

The higher rates of oxygen consumption (NS) and cytochrome oxidase activity ($P < 0.025$) and the lower respiratory control ratio (NS) coupled with the lower ADP/O ratio ($P < 0.025$) associated with the SO mitochondria imply that these mitochondria have an internal source of ADP which cannot be traced to a difference in MG^{++}-activated ATPase activities (Table III). We propose that the source of ADP can be traced to the higher phospholipase A activities in mitochondria of rats (NS), chicks ($P < 0.01$) and guinea pigs ($P < 0.05$) which were fed the SO diet. The fatty acid profiles of the mitochondrial lipids (e.g., rats, Table II) exhibit disproportionate quantities of unsaturated fatty acids. The perturbation of the fatty acid component of the membrane phospholipids requires remodeling which underlies the initiation of a corrective phospholipase activity. The activation of intramitochondrial free fatty acids preceding β-oxidation or esterification of the membrane lysophosphatide requires GTP, the regeneration of which requires an ATP expenditure. The data shown in Table III are consistent with this suggested action.

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The Influence of Linoleic Acid Intake on Electron Transport System Components

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ABSTRACT

Groups of young rats were fed a basal diet with beef tallow (BT) or corn oil (CO) added to provide 4 or 19% energy as linoleic acid. Mitochondria isolated from the livers of the rats fed the CO contained a significantly lower concentration of b-type cytochrome and significantly higher concentrations of cytochromes c, c_1 and aa_3. Cytochrome c oxidase activity also was elevated. The spectral characteristics of the b-type cytochrome varied between the 2 groups. The mitochondria from the rats fed CO contained relatively more of the cytochrome b-558 component whereas mitochondria from the BT group contained more of the cytochrome b-562 component. The classical antimycin A inhibition of electron transport between cytochromes b and c_1 was partially bypassed in mitochondria with the more fluid membrane. The activation energy for cytochrome c oxidase in mitochondria from this group was significantly higher. These differences may be traced to the physical characteristics of the inner mitochondrial membrane.

INTRODUCTION

The nature and extent of the hydrophobic bonding between phospholipids and protein in a lipoprotein complex determines its physical stability and the orientation of the constituent protein molecules (1). Of the cellular phospholipids, the phospholipids located in the hepatic mitochondrial membrane are most sensitive to the influence of dietary fats (2). When essential fatty acids are replaced by nonessential fatty acids, the mole percentage of unsaturated fatty acids in subcellular particles of rat liver remains fairly constant due to increased 16:1, 18:1 and 20:3ω9 synthesis, but the double bond indices fall (3). Changes in the physical state of the membrane lipids then contribute to the development of the symptoms of deficiency. The adaptive lipogenic responses to fat-free and essential fatty acid-free diets might be considered a futile homeostatic effort to maintain the substrate supply required for membrane maintenance.

Ingestion of diets rich in fat (4), particularly in linoleate (5,6), markedly inhibits fatty acid synthesis. A dietary fat patterned after current dietary guidelines would effectively limit the supply of substrates for phospholipid synthesis to those acyl moieties present in the diet. A diet high in linoleic acid, therefore, would increase the double bond index of the mitochondrial membrane lipids.

We recently examined respiratory activities in hepatic mitochondria taken from rats fed 4 or 24% energy as linoleic acid (7).

Although cytochrome oxidase activity was significantly higher under the 24% linoleic acid dietary conditions, the ADP:O (P:O) ratio in response to succinate was significantly lower. These results suggested to us that an excess as well as a deficiency of dietary linoleic acid causes a perturbation of the mitochondrial membrane leading to a disturbance of energy utilization. In the following report, a diet providing 19% energy as linoleic acid is shown to markedly increase cytochrome oxidase activity and to alter the concentrations of the other components of the electron transport system. The permeability of the inner mitochondrial membrane and the kinetic properties of a membrane-bound oxidative enzyme are shown to vary from those observed in mitochondria taken from rats fed a 4% linoleate diet.

MATERIALS AND METHODS

Male Sprague-Dawley rats (218 ± 6.0 g) purchased from ARS-Sprague-Dawley (Madison, WI) were housed individually in mesh-bottomed cages at the Animal Care Unit of the University of Wisconsin Medical School. Palatable diets formulated according to Abuirmelieh and Elson (7) provided 35% energy as fat, 45% as carbohydrate and 20% as protein as a density of 3.1 Kcal/g. Edible beef tallow (BT) (a gift from Oscar Mayer and Company, Madison WI) or corn oil (CO) was added to the diet; the diets as formulated provided 4 or 19% of energy as linoleic acid. Each fat contained 0.03% vitamin E acetate (a gift from Hoffman-LaRoche Inc., Nutley, NJ). Diet and water were given ad libitum for 3 weeks. During the fourth week, the rats were decapitated and the livers were

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immediately excised, washed in a chilled medium of 40 mM Tris-HCl, pH 7.4, 250 mM sucrose and 1 mM EGTA and then homogenized in 8 vol of the medium with a Potter-Elvehjem-type homogenizer. The mitochondria, prepared by differential centrifugation according to Schneider (8), were held at ice temperature. Mitochondrial oxidative activities in response to specific substrates were determined polargraphically as described by Estabrook (9). An aliquot of mitochondria (2 mg protein) was added to the chamber of a Clarke oxygen electrode which contained 1 ml buffer (20 mM KCl, 225 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 20 mM triethanolamine HCl, pH 7.4). Palmitoyl carnitine (1-10 nmol) was added to establish state-4 respiration; then 0.6 μmol ADP was added to establish state-3 respiration. The oxygen concentration in the medium at the time of substrate addition was 0.24 mM and the temperature of the chamber was maintained at 26 C. The data, nmol O_2/min/mg protein, were plotted as double reciprocals with the palmitoyl carnitine concentration.

Cytochrome c oxidase activity of frozen and thawed mitochondrial preparations was measured over a temperature range of 5 and 38 C as described by Wharton and Tzagoloff (10). The logs of the specific activity (nmol reduced cytochrome c oxidized/min/mg protein) were expressed as Arrhenius plots.

The swelling properties of the mitochondria, measured by the initial rate of decrease in absorbance at 520 mμ at 25 C after the addition of 10 mM glutathione, were examined as described by Haeffner and Privett (11).

Difference spectra of fresh mitochondrial preparations were plotted at room temperature and at liquid nitrogen temperature (low temperature) using a split-beam recording spectrophotometer. Sodium hydrosulfite, succinate, oleoyl carnitine and oleoyl CoA plus carnitine served as the reducing agents. Potassium ferricyanide or bubbled oxygen was the oxidizing agent. Protein and substrate concentrations and specific electron transport inhibitors are listed with the results. The concentrations of the individual cytochromes were estimated according to the Williams method (12) which was modified by Williams (13) to incorporate the extinction coefficient for cytochromes aa_3, as proposed by VanGelder and Slater (14,15), and cytochrome c, as proposed by Vanneste (16).

Mitochondrial protein concentrations were determined by the biuret reaction as adapted by Gornall et al. (17) with bovine serum albumin serving as the standard.

Methyl esters of the fatty acids in the extracts of mitochondrial lipids (18) and the dietary fats were prepared according to the method described by Metcalf et al. (19). Gas liquid chromatographic analysis of the fatty acid methyl esters was performed at 165 C on a 0.32 x 183 cm glass U-column packed with 6% diethylene glycol succinate on Chromosorb W (60-80 mesh). The individual methyl esters were identified using retention times of methyl esters in NIH mixture D and quantitation was done by triangulation. Materials and the TBA method were given in the companion report (7).

RESULTS

The fatty acid patterns of the mitochondrial lipids reflect the respective patterns of the dietary fats (Table I). Unsaturated fatty acids in each of the lipid extracts account for 60% of the total fatty acids. The distribution of unsaturated fatty acids, however, varies considerably because monoenes account for 70% of the unsaturated fatty acids in mitochondrial lipids of the BT group but only 22% in the CO group. Although the relative proportions of arachidonic acid and its precursor, linoleic acid, were 2.5-fold higher in the CO group, a 1:1 ratio between the 2 acids was found in each group. Mitochondria from the rats fed the CO diet exhibited a 2.5-fold greater initial rate of swelling than did the BT mitochondria when exposed to 10 mM glutathione (1.5 and 0.6 A/g protein min). When the EGTA was omitted from the medium, the initial rate of swelling was 20-fold greater for the CO mitochondria.

Shown in Figure 1 are representative difference spectra (chemically reduced-chemically oxidized) at room temperature and low temperature of the cytochrome components of mitochondria. At room temperature, the difference spectrum of the BT mitochondria reveals a prominent cytochrome b component, the shoulder appearing at 562 mμ. The cytochrome cc_1 and aa_3 components appear at 552 and 604 mμ, respectively. The CO mitochondria exhibit a less marked cytochrome b component. At low temperature, the cytochrome b is separated from the cytochrome cc_1. The cytochrome b absorption maxima is shifted to 555 mμ and the cytochrome cc_1 maxima to 548 mμ. The difference spectrum of the BT mitochondria cytochromes shows a greater absorption at 555 mμ than at 548 mμ. The reverse relationship is noted in the difference spectrum of the CO mitochondria.

Estimates of the concentrations of the individual cytochromes appear in Table II. For comparison, values recorded by Williams (13) also are shown. While mitochondria from the

TABLE I

Fatty Acid Profiles of Dietary Fats and of Lipids Extracted from Hepatic Mitochondria of Rats Fed the Respective Fats: Vitamin E Content of Diets and Tissue TBA Values

Fatty acid	Dietary fat		Dietary group	
	Beef tallow	Corn oil	Beef tallow	Corn oil
16:0[a]	26.9	12.5	22.8 ± 0.1[b]	21.5 ± 0.9
16:1	4.9		5.2 ± 0.2	3.2 ± 1.5
18:0	23.1	2.5	16.5 ± 0.3	20.2 ± 0.9
18:1	41.8	30.0	37.5 ± 0.9	9.9 ± 0.3
18:2	3.4	54.0	9.3 ± 0.5	23.2 ± 0.8
20:4			8.1 ± 0.5	22.0 ± 0.5
α-Tocopherol:linoleic acid				
Vitamin E, total (mg)	45.1	89.8[c]		
Linoleic acid (g/kg)	14.6	64.6		
Ratio	3.1	1.1		
Thiobarbituric acid number[d]				
Liver			2.92 ± 0.53[b]	3.28 ± 0.30
Heart			1.68 ± 0.68	2.00 ± 1.15

[a]Fatty acids are designated by carbon number and number of double bonds.

[b]SEM, n = 8.

[c]Reference 7 and corn oil, 102 mg total; 12.6 mg α- and 89.4 mg γ-tocopherol/100 g (γ-tocopherol potency, 0.1 α tocopherol). See ref. 20.

[d]Mg of 1,1,3,3-tetraethoxypropane/kg tissue.

BT group contain more of the cytochrome b component ($P < 0.01$), mitochondria from the CO group contain more of the c_1, c and aa_3 components of the electron transport chain ($P < 0.05$). Ratios of the molar concentrations of cytochromes b, c_1 and c to the concentration of cytochrome aa_3 also are shown in Table II. The ratios indicate that the relative concentration of cytochrome b is indeed lower in mitochondria of the group fed the corn oil. This relative decrease in the cytochrome b component was observed also when succinate or oleoyl carnitine served as the reducing agent and bubbled oxygen as the oxidizing agent.

There is evidence for the existence of 2 distinct species of b-type cytochromes in rat liver mitochondria based on measurement of the midpoint oxidation-reduction potentials (Em). One of the b-type cytochromes has variable Em values depending on the energy state ([ATP]/[ADP][Pi] ratio) of the mitochondria. This component has double α peaks at 566 mμ (α_1) and 558 mμ (α_2), the 558 mμ peak appearing as a shoulder (Wilson and Dutton (21), Sato et al. (22)). This component is thought to be directly responsible for energy transduction (T). The other component of the b-type cytochromes is the classical cytochrome b having a fixed Em value. This cytochrome was named cytochrome b_K in honor of Professor David Keilin (Chance et al. [23]). Cytochrome b_K exhibits an absorption peak at 562 mμ. The flow of electrons is believed to pass from the substrate sequentially through cytochrome b_T and cytochrome b_K to cytochrome c_1 (Wikström (24)). Combinations of these b-type cytochromes are located in complex III of the electron transport system.

As the CO diet appeared to specifically decrease the relative concentration of cytochrome b, we undertook a series of spectral studies to more clearly define this response. For this series of studies, rotenone (rot), malonate (malon), antimycin A (ant A) and cyanide (CN^-) were employed as required to block the

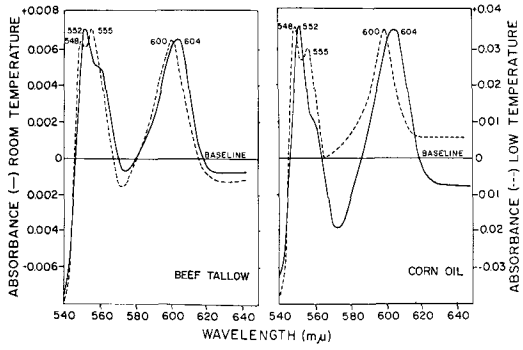

FIG. 1. Difference spectra of cytochrome components of mitochondria determined at room (—) and liquid nitrogen (----) temperatures. Reduction and oxidation were effected by the addition of a few grains of sodium hydrosulfite and potassium ferricyanide to the sample and references cuvettes, respectively.

TABLE II

Concentrations of Respiratory Chain Cytochromes[a] in Mitochondria Isolated from Livers of Rats Fed Diets Supplemented with Beef Tallow or Corn Oil

Cytochrome	Williams[b]	Diet group	
		Beef tallow	Corn oil
	nmol cytochrome/mg mitochondrial protein		
b	0.280 ± 0.005 (1.04)[c]	0.294 ± 0.016[d,e] (1.12)	0.251 ± 0.018 (0.85)
c_1	0.170 ± 0.006 (0.63)	0.180 ± 0.008[f] (1.69)	0.207 ± 0.012 (0.70)
c	0.210 ± 0.005 (0.78)	0.185 ± 0.015[f] (0.71)	0.214 ± 0.010 (0.72)
aa_3	0.270 ± 0.005	0.262 ± 0.010[f]	0.297 ± 0.014
Total	0.930	0.921	0.969

[a]The difference spectra of the cytochrome components were traced using a split-beam spectrophotometer. Each cuvette contained 3 ml oxygen buffer and 6 mg mitochondrial protein. Sodium hydrosulfite was added to the sample (reduced) cuvette and potassium ferricyanide to the reference (oxidized) cuvette after the baseline was established.

[b]Values reported by Williams (13).

[c]Molar ratio with cytochrome aa_3.

[d]SEM, n = 8.

[e]$P < 0.01$ between groups.

[f]$P < 0.05$ between groups.

respective oxidations of $NADH+H^+$, succinate, cytochrome b and cytochrome aa_3 in the sample and/or reference suspensions of mitochondria.

The spectra indicated by the dashed line in Figure 2 indicates that the impermeance of the inner mitochondrial membrane to the oleoyl CoA thioester remained intact. Then, following the sequential additions of ant. A. CN^- and carnitine (Cn), the difference spectra of the BT and CO mitochondria were traced (solid line). The absorption maxima for the cytochrome b of the BT mitochondria was at 556 mμ; for the CO mitochondria, the maximal absorption occurred at 564 mμ. Cytochromes cc_1 and aa_3 were not reduced in the presence of the transport inhibitors. Repetitive scannings were recorded. After 10 min, there was a shift of the peaks to 562 (BT) and 559 (CO) mμ. A marked absorption also was present in the cytochrome aa_3 area of the spectrum for the CO mitochondria.

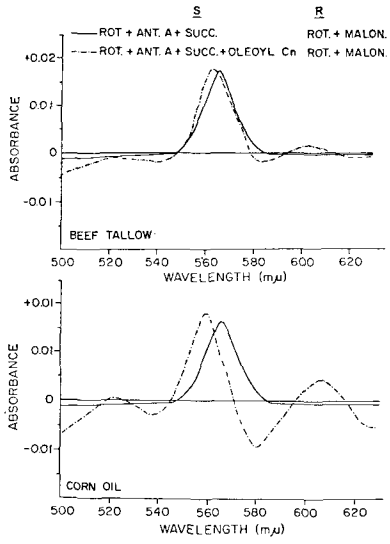

FIG. 2. Difference spectra, determined at room temperature, of the cytochrome b component of mitochondria.

For the next study (Fig. 3), the oxidation of endogenous $NADH+H^+$ by the mitochondrial suspension in the sample cuvettes was inhibited with rot. and to the reference cuvettes, rot. and malon. were added. Then, ant. A and succinate were added to the sample cuvettes. The spectra traced for each mitochondrial preparation exhibit a single cytochrome b peak at 566 nm. Ant. A effectively inhibited the forward flow of electrons arising from succinate oxidation. Following the addition of oleoyl Cn to the sample cuvettes, the spectra were shifted as already described with absorption maxima appearing at 562 (BT) and 559 (CO) mμ. Cytochrome aa_3 of the CO mitochondria was notably reduced in the presence of ant. A by oleoyl Cn but not by succinate.

For the fourth spectral study (Fig. 4), the mitochondrial suspensions in both the samples and reference cuvettes were reduced with succinate. Ant. A and ADP were then added to

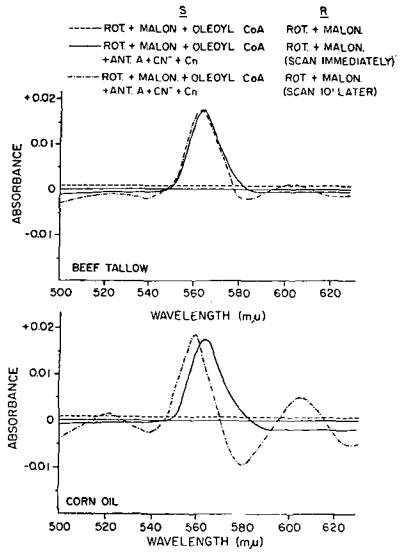

FIG. 3. Difference spectra of the cytochrome b component of mitochondria. The cytochromes were sequentially reduced with succinate (—) and then oleoyl carnitine (----).

the sample cuvette to effect the oxidation of cytochromes cc_1 and aa_3 and to maintain cytochrome b in the reduced state. The absorption minima at 551-552 and 605 mμ represent the oxidized cytochromes. After 10 min, palmitoyl Cn was added to the sample cuvette and repetitive scannings were made. Within 1 min, the cytochrome cc_1 was partially reduced, particularly in the CO mitochondria (dashed line). These spectral studies demonstrate that the electron flow from fatty acid oxidation, but not from succinate oxidation, proceeds partially in the presence of ant. A, the classical inhibitor of the flow between cytochrome b and c_1. The greater flow of electrons past this inhibitory site observed in the CO mitochondria might be traced to the more fluid nature of this inner membrane.

Lineweaver-Burke plots of the oxidation of palmitoyl Cn by the mitochondria preparations are shown on Figure 5. The maximal rate of oxygen uptake by mitochondria of the CO group was 20% higher than that calculated for mitochondria of the BT group (28.2 and 23.5 nmol/min/mg protein) as calculated from the intercept points. The K_m for both groups was 5.55 μM for palmitoyl Cn. Thus, the catalytic rate, but not the affinity of the oxidative system for palmitoyl Cn, is elevated in mitochondria of the CO group relative to the rate of the BT group. The more fluid, lipid environment of the inner membrane of the CO mitochondria must facilitate the transport or oxidative process by influencing the tertiary structure of the involved protein.

Raison et al. (25) suggested that the discontinuity in Arrhenius plots of the respiratory enzyme system is due to configurational changes in the enzyme proteins induced by a temperature-dependent phase change in the mitochondrial membrane. The specific activity (sp act) of the rat liver cytochrome c oxidase was studied as a function of temperature. The results are expressed as Arrhenius plots (Fig. 6). The plots show distinct discontinuities at 13 C (CO) and 15.4 C (BT). The activation energies (Ea) above the transition points were 8.15 (CO) and 6.54 (BT) Kcal/mol. At temperatures

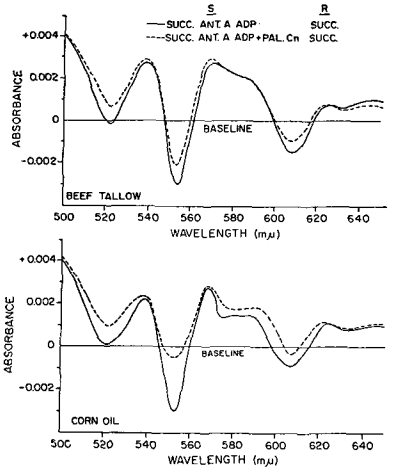

FIG. 4. Difference spectra of the cytochrome cc_1 and aa_3 components of mitochondria.

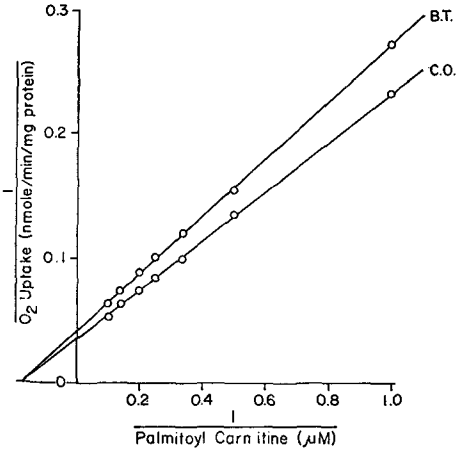

FIG. 5. Linear double reciprocal plots of oxygen consumption vs concentration of palmitoyl carnitine by hepatic mitochondria.

below the transition points, the respective Ea were 22.28 and 20.39 Kcal/mol. The change in Ea is associated with a temperature-induced change in the physical state of the membrane lipids. The transition points for the 2 groups are separated by 2.4 C. At 30 C, the activity in CO mitochondria is 50% greater, and at 38 C, 70% greater than that present in BT mitochondria. Tests of the regression coefficients indicated all values were significant at $P < 0.01$.

DISCUSSION

Fatty acids in animal tissues reflect the fatty acids in the animal's diet. The effect is most pronounced when the diet is high in a dietary fat consisting primarily of polyunsaturated fatty acids. These 2 factors in concert reduce the rate of hepatic fatty acid synthesis; therefore, the influence of these dietary lipids on tissue lipids is magnified (2,26). The physical characteristics of phospholipids and therefore of the membranes in a major part are dependent upon the types of fatty acids present (27,28). The hydrophobic moieties of protein penetrate the lipid core of the inner mitochondrial membrane forming a loose network which is cemented by a discontinuous lipid bilayer (29,30). This penetration is facilitated by the presence of loosely packed acyl chains of unsaturated fatty acids, in particular, arachidonic acid (31). The activity of enzymes existing in such an environment is dependent upon the fluidity of the lipoprotein complex, a property influenced solely by the one changeable component, the fatty acids. Such effects have been demonstrated in vitro by reconstituting lipid-depleted mitochondria (32) and in vivo with essential fatty acid-deficient diets (33). We have now demonstrated that the activities of membrane-bound enzymes increase concomitantly with the degree of unsaturation of the component fatty acids in the membrane.

The possibility exists that the effects attributed to increased membrane fluidity are, in fact, due to a weakened, more permeable membrane, the consequence of a functional vitamin E deficiency. The factors listed in the following lead us to discount this possibility. The CO diet provided vitamin E at the relative ratio of 1.1 mg/g linoleic acid (Table I), a value which indicates a margin of safety (34). The TBA values recorded in Table I for tissues from each group of rats are similar. Experimentally, differential swelling rats for the CO mitochondria were shown in the presence and absence of EGTA. The clearest evidence of the intactness of the membranes is shown on Figure 2. Oleoyl CoA in the absence of Cn was not

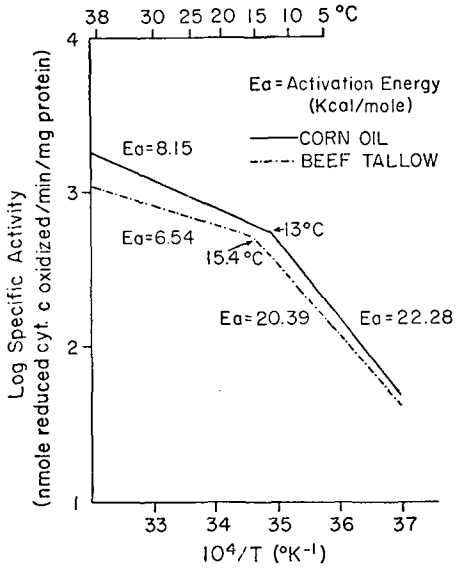

FIG. 6. Arrhenius plots of the activities of cytochrome c oxidase in frozen and thawed mitochondria.

oxidized; upon the addition of Cn, the substrate entered the mitochondria.

The puzzling result of this study is the influence of dietary fat on the cytochrome b component of the electron transport system. The quantity of this cytochrome was significantly reduced in response to the CO diet; the spectral characteristics of the cytochromes of CO origin were similar to those of the BT origin when reduction was effected by succinate in the presence of ant. A. When reduction was affected by the oxidation of an acyl Cn, differences in the spectral characteristics of the b-type cytochromes of different dietary origins surfaced. Our interpretation is that in the CO mitochondria, the cytochrome b-566 and b-562 were equally reduced to form an absorption maxima at 564 mμ. As electrons were transported past the ant. A block to cytochrome c_1, the cytochrome b-558 became the major reduced species of the b-type cytochromes. The mitochondria of the BT group, when reduced by an acyl Cn exhibited a cytochrome b-566. With time, and with little reduction of cytochrome c_1 below the ant. A block, the cytochrome b-562 developed as the major reduced cytochrome b species. Changes in the fluidity of the lipoprotein matrix in which the components of the electron transport system are embedded must be causally related to these activities.

Animals fed dietary fats of high linoleic acid content exhibit mitochondrial lipids disproportionately high in polyunsaturated fatty

acids. In a companion report (7), we suggested that the peturbed state of these mitochondrial membranes initiates remodeling responses which are secondarily accompanied by greater mitochondrial oxidative activity. We have confirmed this observation of increased oxidative activity. Data presented in this report indicate that fatty acyl CoA, the oxidation of which proceeds through one of 3 flavin-linked dehydrogenoses, depending upon the carbon chain length and degree of unsaturation (30), serves as the substrate for the increased oxidative activity. The flavin-linked dehydrogenases are linked to the respiratory chain through a soluble electron transfer flavoprotein (30). There is evidence that the electron transport flavoprotein is connected at more than one site to the respiratory chain (35). Results we obtained using ant. A, the classical inhibitor of electron transport between cytochrome b and cytochrome c, suggest that the electron transport flavoprotein is linked functionally to both sites. Mitochondrial membranes rich in polyunsaturated fatty acids permit the entry of these mitochondria, the concentrations of the individual components of the b-type cytochrome are altered, perhaps to accomodate the increased oxidative activity.

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Gangliosides of Bovine Optic Nerve

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ABSTRACT

Gangliosides from bovine optic nerve were analyzed. The optic nerve contained 129, 98, 97, 80, 31 and 12 μg of GM_1, GD_{1a}, GD_{1b}, GT_{1b}, GD_3 and GQ_1 gangliosides, respectively, per g of tissue wet wt. These 6 gangliosides altogether contributed 97% of the total sialic acid. GM_3 and GM_2 gangliosides contributed the remaining 3% of total sialic acid. Stearoyl (18:0) was the predominant acyl group (61-76%) in all gangliosides. There was a marked variation in acyl group composition between GT_1 and most of the other major gangliosides except GD_3. In comparison to the other gangliosides, GT_1 contained a lower proportion of the stearoyl group and a higher proportion of the oleoyl, nervonoyl and the long chain acyl groups. Both GT_1 and GD_3 gangliosides contained proportionately higher levels of long chain acyl groups (20:0 → 24:0) than did other gangliosides. GD_3 gangliosides showed 2 bands on thin layer chromatography, and the upper band was more distinct than the lower band.

Many studies have dealt with the ganglioside composition of tissue associated with central nervous system, such as retina (1-3) and brain (4-12), because of the possibility that gangliosides are dynamically involved in various functions of these tissues. Gangliosides have been isolated from human femoral nerve (13) and animal peripheral nerves (14-15). Lowden and Wolfe (6) did not succeed in isolating gangliosides from human optic nerve nor from ox sciatic nerve. Forman and Ledeen (16) reported there is a rapid axonal transport of gangliosides in the optic nerve of goldfish. Holm (17) detected gangliosides in the optic nerve of rabbits, but did not find any axonal transport. Holm and Mansson (18) isolated gangliosides from bovine optic nerve; however, they did not present quantitative data for the ganglioside composition. It is surprising that even though the optic nerve is an integral part of the central nervous system and links the retina and brain, its ganglioside composition has not been studied in detail. This study aims to investigate in detail the distribution of gangliosides and their fatty acid composition pattern in bovine optic nerve.

MATERIALS AND METHODS

Tissues

Fifty bovine eyes were obtained from 3-4-year-old-male animals at slaughter, transported on ice to the laboratory, and the optic nerves were removed and processed immediately.

Ganglioside Extraction

Optic nerves, ca. 250-270 mg/eye, were pooled from 50 eyes into 5 samples. Ganglioside was extracted from the wet tissue by the Folch-Suzuki-Partition procedure as described by Brunngraber et al. (19) with the following modifications: optic nerve was homogenized in a glass tissue grinder for 5 min with 19 vol of chloroform/methanol (2:1, v/v); after centrifugation, the residue was treated with 10 vol of chloroform/methanol (1:2, v/v) containing 5% water. Both supernatants were combined and an appropriate amount of chloroform was added to yield a final ratio of chloroform/methanol of 2:1 (v/v). Gangliosides were partitioned into the aqueous upper phase by the addition of 0.2 vol of 0.94% KCl. The lower phase was washed once with the Folch theoretical upper phase containing KCl (chloroform/methanol/0.88% KCl, 3:48:47, v/v/v), and once more with the upper phase containing no salt. The combined upper phases were evaporated to dryness with a stream of nitrogen. The residue was mixed with 0.5 ml of 0.6 N methanolic NaOH and 0.5 ml of chloroform, and incubated at 37 C for 1 hr for mild alkaline hydrolysis (20). The hydrolysate was neutralized with conc HCl, evaporated to dryness under nitrogen, dissolved in water and dialyzed against cold demineralized water at 4 C for 24 hr in a bag treated with Na_2CO_3 and EDTA (21). The contents of the dialysis bag were lyophilized. The residue was dissolved in a small known vol of chloroform/methanol (1:1, v/v), the solution mixed with a sufficient vol of chloroform to bring the solvent ratio to 85:15, and then the mixture applied to a 12 × 150 mm chromatography column containing Bio-Sil A, 200-325 mesh silicic acid (Bio-Rad Laboratories, Richmond, CA) (22). Gangliosides were separated from contaminants such as sulfatides, and fatty acids by successive use of 2 solvents, chloroform/methanol (85:15, v/v), and chloroform/methanol (2:3, v/v) as described by Yu and Ledeen (22). Gangliosides were eluted in the pure form with the second solvent system.

Analysis of Gangliosides

Aliquots of ganglioside samples and reference standards (GM_2, GM_1, GD_{1a} and GT_1 from Supelco, Inc., Bellefonte, PA, GM_3 from dog erythrocytes and mixed gangliosides from human brain), containing 20-30 μg sialic acid (N-acetylneuraminic acid or NANA) were subjected to thin layer chromatography (TLC) on glass plates coated with 250-μm-thick Silica Gel 60 (EM Laboratories, Elmsford, NY). Chloroform/methanol/0.02% $CaCl_2$ (50:40:10, v/v/v) was used as the solvent system for the separation of gangliosides (21). The resorcinol reagent (23), diluted with an equal vol of water, was used to detect gangliosides. For the quantitative analysis of sialic acid, sugars and fatty acids in the gangliosides, major individual ones were isolated by preparative TLC. The bands were located by brief exposure of the plate to iodine vapor. After complete disappearance of the yellow color of iodine, the lipid spots were scraped from the plate and extracted from the gel by a small known vol of chloroform/methanol/water (10:10:2, v/v/v) (2). To confirm the identity of minor ganglioside species, a second solvent system, chloroform/methanol/ammonia/water (60:35:1:7, v/v/v) (24) also was used.

Lipid-bound sialic acid in the ganglioside fraction was estimated by Svennerholm's procedure (25) as modified by Miettinen and Takki-Luukkainen (26). Data were expressed as nmol sialic acid/g tissue wet wt. No attempt was made to differentiate N-acetylneuraminic acid (NANA) vs N-glycolylneuraminic acid (NGNA) in the total sialic acid.

The amount of sugars (hexose + hexosamine) in the individual ganglioside fractions were determined by the phenolsulfuric acid assay method (27). Data were expressed as nmol total sugars/g tissue wet wt. *D*(+) - galactose was used as standard for the colorimetric analysis of the carbohydrates. No attempt was made to characterize individual sugar species in any major gnagliosides. However, the sugar composition of 2 minor components, GD_3 and GQ_1, was determined by subjecting the trimethylsilyl ethers of the *o*-methyl glycosides to gas liquid chromatographic (GLC) analysis as described by Vance and Sweeley (28). The analyses were performed in a Packard model 7400 gas chromatograph equipped with a flame ionization detector and a glass column (6 ft × 1/4 in. od) packed with 3.0% SE-30 on 80/100-mesh Supelcoport. The instrument was programmed from 120 to 180 C at 5 C/min and a nitrogen flow rate of 30 ml/min.

The fatty acid pattern of individual ganglioside fractions was determined by following the Vance and Sweeley procedure (28). Lipid residues, containing ca. 0.4-0.5 μmol of fatty acids, were subjected to methanolysis with 1 ml of 0.75 N methanol/HCl. 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 (8). Normal fatty acid esters were separated on a 6 ft × 1/4 in. column of 10% DEGS-PS on 80/100 Supelcoport in a Packard Model 7400 gas chromatograph equipped with a flame ionization detector. The methyl esters were analyzed with a temperature program of 10 C/min from 120 to 200 C and at a nitrogen flow rate of 35 ml/min. Assignments for each peak were obtained from a semilogarithmic plot of relative retention time vs chain length and degree of unsaturation of standard mixtures of fatty acid methyl esters. Peak areas were determined by multiplying the height by the width at half-height. The relative percentage of each fatty acid methyl ester was determined by dividing the area under individual peaks by the total peak area.

For further characterization of the minor gangliosides, GD_3 and GQ_1, each sample containing ca. 0.1 μmol of sialic acid was dissolved in 1 ml of 0.1 M acetate buffer (pH 5) containing 6.58 mM $CaCl_2 \cdot 2H_2O$ (22). A small vol of neuraminidase from *Clostridium perfringens* (type VI, Sigma Chemical Co., St. Louis, MO) containing 0.1 unit of activity was added. The solution was covered with 0.2 ml of toluene and incubated at 37 C for 16 hr. After 2 hr of incubation, a further addition of 0.1 enzyme unit was made. The toluene was removed with a stream of nitrogen and the reaction mixture was treated with 20 vol of chloroform/methanol, 2:1. The resulting precipitate was removed by filtration, and the filtrate was evaporated to dryness. The lipids were redissolved in a small vol of chloroform/methanol (1:1, v/v). Aliquots of this solution, untreated ganglioside and reference standards (GM_1 from Supelco, Inc., and bovine spleen lactosyl ceramide, a gift from S. Basu of the University of Notre Dame) were subjected to TLC using chloroform/methanol/0.02% $CaCl_2$ (50:40:10, v/v/v) as the solvent system. Resorcinol reagent (23) diluted with an equal vol of water was used to detect spots. The identity of lactosyl ceramide, as obtained after TLC separation of the neuraminidase- (Sialidase) treated solution, was confirmed by sugar analysis by GLC (28).

RESULTS AND DISCUSSION

Gangliosides isolated from bovine optic nerve were separated into 4 major fractions on TLC using the solvent system of Coleman and

Yates (21) (Fig. 1A). The nomenclature used by Svennerholm (4) was adopted for this study of the ganglioside characterization. These 4 fractions were identified as the gangliosides GM_1 (R_f = 0.62), GD_{1a} (R_f = 0.48), GD_{1b} (R_f = 0.30) and GT_{1b} (R_f = 0.18). In addition, 6 more minor components were detected. Two of these had the same R_f as those of GM_3 (R_f = 0.85) and GM_2 (R_f = 0.67). Three components had the same R_f as those of GD_3 (R_f = 0.51), GD_2 (R_f = 0.42) and GQ_1 (R_f = 0.11) fractions from human brain. The other component (R_f = 0.54) was distinct in optic nerve but not in human brain.

The presence of minor ganglioside components were confirmed by TLC with a second solvent system, chloroform/methanol/conc NH_3/water (65:35:1:7, v/v) (24) (Fig. 1B). The minor components, GD_3 and GQ_1, also were characterized by GLC determination of their sugar composition. The intact GD_3 gangliosides contained only glucose and galactose in the molar proportions 1:1. GQ_1 gangliosides contained glucose, galactose and N-acetylgalactosamine inthe molar proportions of ca. 1:2:1.

Data on the composition of gangliosides are given in Table I. The optic nerve contained 478 nmol sialic acid/g wet wt. of tissue. This value is in agreement with that of Holm and Mansson (18), and is considerably higher than that found in peripheral nerve. Altogether, the GM_1, GD_{1a}, GD_{1b} and GT_1 gangliosides contained 81.3% of the total sialic acid. The minor gangliosides, GD_3 and GQ_1, altogether accounted for 15.5% of the total sialic acid.

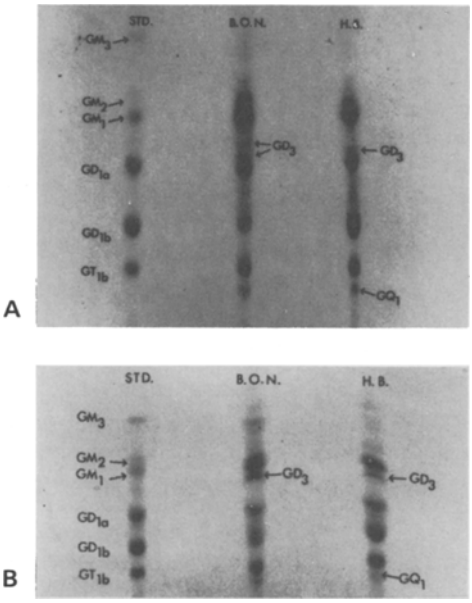

FIG. 1(A). Thin layer chromatograms of the gangliosides of bovine optic nerve (BON) and human brain (HB). Channel 1 contained a mixture of standard gangliosides; channel 2 contained BON gangliosides; and channel 3 contained HB gangliosides. The chromatogram was developed in chloroform/methanol/0.02% $CaCl_2$ (50:40:10) by vol and sprayed with resorcinol reagent. (B) Thin layer chromatograms of the gangliosides of bovine optic nerve (BON) and human brain (HB). Channel 1 contained a mixture of standard gangliosides; channel 2 contained BON gangliosides; and channel 3 contained HB gangliosides. The chromatogram was developed in chloroform/methanol/conc NH_3/water (65:35:1:7) by vol, and sprayed with resorcinol reagent.

TABLE I

Composition of Bovine Optic Nerve Gangliosides

Components	Lipid-sialic acid[a]	Lipid = (hexose + hexosamine)[a]	Hexose + hexosamine[b] / sialic acid	Gangliosides[c]
Total	478 ± 14	Not determined		
GM_1	81 ± 3	313 ± 5	3.9 ± 0.2	129 ± 4
GD_{1a}	103 ± 3	200 ± 4	1.9 ± 0.1	98 ± 2
GD_{1b}	102 ± 2	196 ± 4	1.9 ± 0.1	97 ± 2
GT_1	103 ± 3	134 ± 4	1.3 ± 0.1	80 ± 2
GD_3	55 ± 6	29 ± 3	1.1 ± 0.0	31 ± 2
GQ_1	20 ± 2	18 ± 2	0.9 ± 0.1	12 ± 2

[a]Each value is the mean (nmol/g wet tissue) ± SE of 5 observations of 10 samples each.

[b]Mean molar ratio ± SE of 5 observations.

[c]Each value is the mean (μg/g wet tissue) ± SE of 5 observations.

Calculation was based on the data obtained for NANA, and assumptions that each mole of major gangliosides (GM_1, GD_{1a}, GD_{1b} and GT_1b) and minor ganglioside GQ_1 contained 1 mol stearic acid, 2 mol galactose, 1 mol glucose, 1 mol N-acetylgalactosamine, and 1 mol sphingosine. For GD_3, calculation was based on the composition that each mol contained 1 mol stearic acid, 1 mol glucose, 1 mol galactose and 1 mol sphingosine.

Although the other minor gangliosides (GM_3 and GM_2) were not analyzed, these probably accounted for much of the remaining 3.0% of the sialic acid. The authenticity of individual major gangliosides, as well as that of GD_3 and GQ_1, was established from the values obtained for molar ratios of sialic acid to carbohydrate (hexose + hexosamine). It is obvious that GM_1, GD_{1a}, GD_{1b}, GT_1, GD_3 and GQ_1 contained 1, 2, 2, 3, 4 and 4 mol of sialic acid/4 mol of carbohydrate, respectively. Based on the assumption that each mol of the major ganglioside (GM_1, GD_{1a}, GD_{1b} and GT_{1b}) and the minor ganglioside, GQ_1 contained 1 mol of stearic acid, 2 mol of galactose, 1 mol of glucose and 1 mol of N-acetylgalactosamine, and 1 mol of sphingosine, optic nerve contained 129, 98, 97, 80 and 12 μg of GM_1, GD_{1a}, GD_{1b}, GT_1 and GQ_1 gangliosides, respectively, per g of tissue wet wt.

When the gangliosides occupying bothspots (R_f 0.51 and R_f 0.54) were incubated with neuraminidase, they were transformed to a resorcinol-negative glycolipid, which moved like the authentic sample of lactosylceramide. Both neuraminidase-treated ganglioside samples and authentic lactosylceramide gave 2 bands (R_f 0.94–R_f 0.96). Furthermore, the GLC analysis of the resorcinol-negative glycolipid as separated by TLC showed that this glycolipid contained glucose and galactose as the only sugar species and the molar ratio of glucose and galactose was 1:1. The released sialic acid had the same R_f as that of authentic NANA (0.08). Neuraminidase treatment of the other minor component, GQ_1, gave 2 resorcinol positive spots, one corresponding to GM_1 and the other corresponding to NANA.

Based on the assumption that each mol of GD_3 ganglioside contained 1 mol of stearic acid, 1 mol of glucose, 1 mol of galactose and 1 mol of sphingosine, optic nerve contained 31 μg of GD_3 ganglioside. However, it is to be noted that these values may not reflect true concentrations because of variation in recovery from silica gel scrapings. For example, monosialogangliosides generally are eluted in better yields than di- and trisialo species.

The optic nerve contains the myelinated axons of the retinal ganglion cells. GD_3 gangliosides constitutes ca. 50% of total gangliosides in the retina (2). Holm and Mansson (18) failed to detect any GD_3 gangliosides in bovine optic nerve, and thus postulated that GD_3 in retina is localized in cells other than ganglion cells, and that the retinal GD_3 cannot be transported through the optic pathway. However, in our study, we are reporting the presence of GD_3 gangliosides in bovine optic nerve, and therefore, the postulation by Holm and Mansson becomes controversial. It is to be noted that Holm and Mansson have not presented any photograph of TLC separation. It might be that they have overlooked the existence of GD_3. Furthermore, Holm and Mansson (18) did not report the presence of any tetrasialogangliosides (GQ_1) in bovine optic nerve. In contrast, we have found a distinct spot of GQ_1 gangliosides in bovine optic nerve, similar to that of human brain. It should be noted that this spot (R_f = 0.11) was different from that of free sialic acid (R_f = 0.08). Polysialogangliosides, such as tetra- and pentasialogangliosides also have been detected in mammalian brain by other workers (4,29-30), in brains of fish and amphibia (9-10) and in human cerebrospinal fluids (31).

GM_1, GD_{1a}, GD_{1b} and GT_1 also have been reported to be the major gangliosides of other tissues, such as rat brain myelin (7), human femoral nerve (13), sciatic nerve of rabbit (14) and rat (15), and human brain (23). Siddiqui and McCluer (8) isolated a sialogalactosylceramide (GM_4) from human brain and reported that this has no direct relationship to the major brain gangliosides. This ganglioside was not detected in bovine optic nerve, perhaps because of the possibility that most GM_4 does not partition into the upper phase, although we have observed a small amount of GM_4 in the human brain sample.

The data on the acyl group composition of individual major gangliosides are shown in Table II. Palmitoyl (16:0), stearoyl (18:0), oleoyl (18:1), arachidoyl (20:0), docosanoyl (22:0), tricosanoyl (23:0), lingoceroyl (24:0) and nervonoyl (24:1) were the major acyl groups detected in all gangliosides. The predominant acyl group in all fractions was the stearoyl group, which constituted 61-76%. There was a variation in the acyl group composition between GT_1 and other gangliosides. For example, the concentration of unsaturated acyl groups (oleoyl and nervonoyl) was higher in GT_1 than other gangliosides. GT_1 gangliosides contained a lower proportion of palmitoyl and stearoyl groups than did other gangliosides. GT_1 and GD_3 gangliosides contained proportionately higher levels of the long chain acyl groups (20:0 → 24:0) than did other gangliosides. It must be emphasized that there was no remarkable variation among acyl group composition of GD_{1a}, GD_{1b}, and GM_1. The data obtained on the acyl group composition in this study is for the most part in agreement with those obtained by Holm and Mansson (18).

Raghaven et al. (12) studied the fatty acid composition of gangliosides of rat "membranous sacs." They reported that GD_{1a} gang-

TABLE II

Acyl Group Composition of Optic Nerve Gangliosides

Acyl group[a]	GM_1	GD_3	GD_{1a}	GD_{1b}	GT_1	GQ_1
16:0	4.2 ± 0.1[b]	3.4 ± 0.4	5.0 ± 0.2	4.9 ± 0.3	2.5 ± 0.1	4.4 ± 0.2
16:1	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	0.4 ± 0.0
18:0	71.9 ± 1.1	66.1 ± 2.3	75.8 ± 1.5	73.5 ± 1.8	60.8 ± 1.4	71.9 ± 2.9
18:1	1.5 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.6 ± 0.2	2.9 ± 0.1	1.4 ± 0.1
20:0	6.6 ± 0.1	12.9 ± 1.2	6.1 ± 0.1	7.0 ± 0.3	10.3 ± 0.6	7.8 ± 0.2
22:0	5.4 ± 0.1	7.8 ± 0.6	4.5 ± 0.2	4.8 ± 0.1	8.2 ± 0.6	5.9 ± 0.2
23:0	2.8 ± 0.2	4.0 ± 1.3	2.4 ± 0.1	2.2 ± 0.1	4.2 ± 0.1	2.4 ± 0.1
24:0	3.1 ± 0.1	2.4 ± 0.6	2.5 ± 0.1	2.6 ± 0.1	4.6 ± 0.1	2.9 ± 0.1
24:1	3.9 ± 0.1	2.0 ± 0.6	2.3 ± 0.1	2.9 ± 0.1	5.5 ± 0.2	2.9 ± 0.1

[a]No. of carbons:no. of double bonds.

[b]Each value is the mean (percent of total acyl groups) ± SE of 5 observations.

liosides are composed mostly of saturated fatty acids (93.3% stearic acid, 3.8% arachidic acid and 2.9% palmitic acid). Holm et al. (2) reported data on the fatty acid composition of retinal and brain gangliosides of humans and rabbits. Even though these authors claimed that there was no remarkable difference in the ganglioside fatty acid composition between retina and brain, their own data reveal that a marked difference exists between these 2 tissues. For example, brain ganglioside contained more of the stearoyl group than did the retinal ganglioside. The fatty acid composition of bovine optic nerve gangliosides, as observed in this study, shows more resemblance to that of the retinal gangliosides than to that of brain as reported by Holm et al. (2). However, it remains to be seen whether there is a difference in the ganglioside fatty acid composition between optic nerve, retina and brain within the same species. Furthermore, the acyl group composition of GT_1 and GD_3 is markedly different from that of other gangliosides. Nothing is known at the present time about the localization of GT_1 or GD_3 gangliosides in optic nerve, although it has been postulated that GT_1 is highly localized in axolemma of peripheral nerve (32).

This work has demonstrated the existence of 2 distinct GD_3 gangliosides from bovine optic nerve. In contrast, the human brain shows the existence of only the lower GD_3 band. The difference in the TLC mobilities of the 2 GD_3 bands from bovine optic nerve most likely results from variations in the fatty acid composition. Further investigation of their fatty acid spectra currently in progress should determine if, indeed, that is the case. The question remains why the optic nerve contains 2 GD_3 components whereas the brain apparently has only one. Are their functional roles identical or different? Is it a species variation or a tissue difference for different functions? What functional roles do the gangliosides have, and can these be determined? These questions and others await further investigation.

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Effects of Coconut Oil on Heart Lipids and on Fatty Acid Utilization in Rapeseed Oil

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ABSTRACT

Male adult Sprague-Dawley rats were fed diets containing 15% by weight of sunflower oil, coconut oil, rapeseed oil or combinations of these oils for 5 or 60 days. The digestibility of erucic acid (22:1), lauric acid (12:0) and linoleic acid (18:2) was measured and found to be decreased for erucic acid at both time intervals, and for lauric acid after 60 days when coconut oil and rapeseed oil were blended. The cardiac lipodosis was proportional to the content of erucic acid in the diet. At 60 days, the high level of 22:6 in the cardiac phospholipids of rats fed rapeseed oil was reduced by the addition of sunflower oil but not by coconut oil. Thus, the blending of rapeseed oil with coconut oil apparently is less desirable than that of rapeseed oil and sunflower oil.

INTRODUCTION

The pathogenic effects on myocardium of feeding rapeseed oils containing high levels of erucic acid are well established (1-6). However, while cardiac lipidosis is related to erucic acid content, this is not true of myocardial lesions since these have also been observed during prolonged feeding of low erucic rapeseed oils. Several hypotheses have been proposed to explain this phenomenon; one possible explanation is that the cardiopathogenicity of these oils is associated with their fatty acid composition (5-12). Attention consequently has been given to the importance of unsaturated fatty acids, and recently to the influence of linoleic and linolenic acid content (11,12), and of hydrogenation (10) of rapeseed oils. The role of the low proportion of saturated fatty acids in rapeseed oils also has been investigated previously, and a synergic effect on digestibility of rapeseed in poultry was shown when long chain saturated fatty acids were added to diets containing high levels of erucic acid rapeseed oil (13-16). However, in spite of Abdellatif and Vles' (8) finding that trilaurin aggravates growth depression and myocardic lesions caused by rapeseed oil consumption in the duckling, there have been no studies of the effects on myocardial lipids of diets containing rapeseed oil blended with oils containing short and medium chain fatty acids, as in margarine (17). The purpose of this work was to determine quantitative and qualitative changes in lipid digestibility and in fatty composition of cardiac triacylglycerol (TG) and phospholipids (PL) in rats after 5 and 60 days of feeding diets containing 15% by weight of either rapeseed oil, sunflower oil, coconut oil or blends of these oils. Particular attention was given to the effects of blending rapeseed oil with coconut oil.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats weighing 180 g (Charles River, St. Aubin-les-Elbeuf, France) were housed in stainless steel cages and fed a standard pellet diet (U.A.R., Epinay-sur-Orge, France) and water ad libitum for 7 days. They were then randomized into 6 dietary treatments, 9 animals per treatment (housed 3 per cage) and fed ad libitum test diets for 5 or 60 days. The semisynthetic diets, prepared as described by Rocquelin and Cluzan (18) contained 15% by weight of one of the following oils: sunflower oil (SO), coconut oil (CO), high erucic acid rapeseed oil (RSO) or oil blendings (50:50, v/v), sunflower oil-rapeseed oil (SO-RSO), coconut oil-rapeseed oil (CO-RSO) and sunflower oil-coconut oil (SO-CO). Table I gives the fatty acid composition of the different oils and blends used in this experiment.

Body weight was recorded each day for animals fed diets during 5 days and twice weekly for the feeding period lasting 60 days. Feed consumption, feces weight and feces total fatty acids pooled for each group of 3 rats in the same cage were measured daily throughout the 5-day period before sacrifice.

Lipid Extraction

Fecal lipids were extracted as described by Savary and Constantin (19). After the animals had been killed, the hearts were removed immediately, weighed, washed with ice-cold saline, cut into small pieces and homogenized.

TABLE I

Composition (% by weight) of Fatty Acid Dietary Oils

Dietary oil	SO[a]	CO	RSO	SO-RSO	CO-RSO	SO-CO
Fatty acid						
6:0	–	0.3	–		0.1	0.1
8:0	–	7.0	–		3.5	3.5
10:0	–	6.0	–		3.0	3.0
12:0	–	45.1	–		22.6	22.6
14:0	–	21.5	–	0.1	10.7	10.7
16:0	6.1	8.2	3.5	4.8	5.9	7.2
16:1	0.4	0.1	0.3	0.3	0.2	0.2
18:0	4.4	2.5	1.3	2.9	1.9	3.5
18:1	23.4	7.4	13.7	18.6	10.6	15.4
18:2	64.4	1.9	15.6	40.0	8.7	33.2
18:3	0.5	–	9.2	4.8	4.6	0.2
20:0	–	–	–	–	–	–
20:1	–	–	9.0	4.5	4.5	–
22:0	0.8	–	–	0.4	–	0.4
22:1	–	–	47.4	23.7	23.7	–

[a]SO, sunflower oil; CO, coconut oil; RSO, rapeseed oil; SO-RSO, CO-RSO, SO-CO, 50/50 blends of respective oils.

Total lipids were extracted by the Folch et al. procedure (20).

Lipid Analysis

An aliquot of heart lipids was used to separate TG from total PL by thin layer chromatography (TLC) on 500 μm thick Kieselgel G (Merck, Darmstadt, F.R.G.) plates using light petroleum ether, diethyl ether, acetic acid and methanol (90:20:2:3, v/v) as the developing system. A reference mixture was separated at each side of the plates. After migration, the plates were dried for a few minutes, lipids were protected by a screen and the reference mixture migration alone was revealed using iodine vapor. The bands corresponding to TG and PL were removed, pentadecanoic acid was added as an internal standard and the mixture was transesterified to butyl esters as described previously (21). Butyl esters (22) were preferred to methyl esters, usually used in gas liquid chromatography (GLC), because they allowed a better quantitative analysis of volatile short chain fatty acids of either coconut oil or lipids of rats fed this oil. Butyl esters were separated by GLC on columns of 1.80 m × 2 mm id packed with 20% DEGS on chromosorb 80/100 mesh using a Packard apparatus, model 750, with flame ionization detector. The isothermal condition used for butyl esters of lipids not containing short and medium chain fatty acids was 195 C. For other lipids, programming at 5 C/min from 100 to 195 C and a final isothermal plate at 195 C was used. The carrier gas was N_2 at 50 ml/min.

Calculations and Statistical Methods

An apparent coefficient of digestibility (ACD) of either total or individual fatty acids was calculated in the following manner: ingested quantity - excreted quantity × 100/ingested quantity. Since covariance analysis showed that there was no significant relationship between ingested and excreted lipid quantities and as the ingested quantities were statistically the same for all diets, no corrective factor was used to calculate ACD.

Homogeneity of variances was determined by Bartlett's test and analysis of variance was performed. Classification of means was performed using Duncan's new multiple range test.

RESULTS

Although there was no significant difference in weight gain and feeding efficiency between the different diet groups, differences were observed in fatty acid digestibility as shown in Table II. Total fatty acid digestibility in animals fed diet fats containing RSO were significantly lower than in other diet groups after 5 days. After 60 days, total fatty acid digestibility in RSO and SO-RSO groups became significantly higher than in the CO-RSO group and consequently digestibility coefficients could be arranged in order of decreasing magnitude as CO-RSO<RSO = SO - RSO<SO - CO = SO. After 60 days, 22:1 apparent digestibility in CO-RSO and SO-RSO was not significantly different but was significantly smaller than in RSO. The difference in total

fatty acid digestibility between CO-RSO and SO-RSO resulted mainly from a decrease in medium chain fatty acid digestibility in CO-RSO compared to CO or SO-CO, as shown by the 12:0 digestibility coefficient. On the contrary, no reduction of digestibility of SO fatty acids such as 18:2 was found when SO was blended with RSO or CO although 18:2 digestibility in CO group was significantly lower than in other groups after 5 and 60 days.

Total fatty acid levels in TG and PL of cardiac lipids are shown in Table III. After 5 days, heart TG levels in the RSO group and RSO blend groups were, respectively, almost 5-fold and 3-fold higher than in groups not receiving rapeseed oil, where heart TG level was statistically the same and the lowest of all groups. No significant difference was found in heart TG level between SO-RSO and CO-RSO groups. The excess in the TG level in the RSO group (difference between RSO TG level and average TG level in SO, CO and SO-CO) was about twice as high as the level of excess TG found in RSO blends and was not significantly different from 2, the theoretical value for the ratio of 22:1 in RSO diet to 22:1 in RSO blend diets. After 60 days, lipidosis disappeared from hearts of animals fed erucic acid. There was no significant difference in the level of PL total fatty acid between all groups after 5 and 60 days, but mean values of PL content after 60 days were slightly greater than after 5 days.

The composition of heart TG fatty acid after 5 and 60 days is shown in Table IV. In spite of TG lipidosis being the same in SO-RSO and CO-RSO after 5 days, heart TG 22:1 level was significantly different between both these groups and there was, respectively, 2.5 and 4 times less erucic acid SO-RSO and CO-RSO than in RSO. After 60 days, although TG 22:1 level was lowered to the same extent in the 3 groups by lipidosis disappearance, the difference observed between SO-RSO and CO-RSO was no longer significant. Distribution of heart TG 18:1 showed a partial analogy to that of 22:1. After 5 days, animals fed diets containing RSO had a heart TG 18:1 level significantly higher than in all other groups, and those fed RSO alone had significantly more 18:1 than those fed RSO blends. In RSO and RSO blend groups, the TG 18:1 content decreased with time and after 60 days there was no significant

TABLE II

Apparent Digestibility of Total Fatty Acids and Erucic, Lauric and Linoleic Acids by Rats Fed Either Undiluted or Blended Sunflower, Coconut and Rapeseed Oils

Dietary treatment		SO	CO	RSO	SO-RSO	CO-RSO	SO-CO	SM_c
5 days	TFA	96.76[a]	92.59	88.4[b]	90.07[b]	89.18[b]	96.72[a]	0.72
	22:1			86.93[a]	75.38[a]	77.11[a]		3.26
	12:0		96.2[a]			96.7[a]	99.20	0.50
	18:2	99.50[a]	91.85	98.76[b,c,d]	99.32[a,b,c]	98.32[d]	99.36[a,b]	0.20
60 days	TFA	98.82[a]	95.91[a,b]	91.45[c]	92.84[c]	87.53	96.5[a,b]	0.98
	22:1			87.10	80.34[a]	78.86[a]		1.66
	12:0		97.90[a]			95.70	98.80[a]	0.48
	18:2	99.90[a]	97.14	99.34[a,b,c,d]	99.66[a,b]	99.09[b,c,d]	99.48[a,b,c]	0.22

TFA, total fatty acids; SM_c, common standard error of the means; SO, sunflower oil; CO, coconut oil; RSO, rapeseed oil; SO-RSO, CO-RSO, SO-CO, 50/50 blends of respective oils.

[a-d]In each line, treatments followed by the same letter are not significantly different ($P < 0.05$).

TABLE III

Quantities (mg/g heart) of Total Fatty Acids of Triacylglycerol and Total Phospholipids of Hearts of Rats Fed Either Undiluted or Blended Sunflower, Coconut and Rapeseed Oils

No. days		SO	CO	RSO	SO-RSO	CO-RSO	SO-CO	SM_c
5	TG	2.30[b]	2.33[b]	11.73	7.06[a]	6.56[a]	2.62[b]	0.83
	PL	11.86[a]	11.34[a]	11.34[a]	11.18[a]	11.89[a]	12.06[a]	0.79
60	TG	2.56[a]	3.26[a]	4.95	3.25[a]	3.02[a]	3.42[a]	0.45
	PL	13.67[a]	13.71[a]	14.05[a]	13.93[a]	14.00[a]	13.45[a]	0.52

SM_c, common standard error of the means; SO, sunflower oil; CO, coconut oil; RSO, rapeseed oil; SO-RSO, CO-RSO, SO-CO, 50/50 blends of respective oils.

[a,b]In each line, treatments followed by the same letter are not significantly different ($P < 0.05$).

TABLE IV

Composition of Triacylglycerol Fatty Acids of Hearts of Rats Fed Either Undiluted or Blended Sunflower, Coconut and Rapeseed Oils

Dietary treatment		SO	CO	RSO	SO-RSO	CO-RSO	SO-CO	SM_c
5 days	12:0		$0.23^{a,b}$	0.03^{b}		0.32^{a}	$0.12^{a,b}$	0.07
	14:0	0.04^{b}	0.19^{a}	$0.11^{a,b}$	0.06^{b}	0.44	$0.12^{a,b}$	0.04
	16:0	0.55^{c}	0.63^{c}	1.30^{a}	1.09^{b}	1.56^{a}	0.66^{c}	0.14
	16:1	0.09^{c}	$0.14^{b,c}$	$0.23^{a,b}$	$0.16^{a,b,c}$	0.28^{a}	$0.11^{b,c}$	0.04
	18:0	0.17^{b}	0.14^{b}	0.44^{a}	0.34^{a}	0.33^{a}	0.16^{b}	0.04
	18:1	0.67^{b}	0.69^{b}	3.12	1.99^{a}	1.85^{a}	0.87^{b}	0.23
	18:2	0.69^{b}	0.18^{c}	1.33^{a}	1.52^{a}	$0.51^{b,c}$	$0.52^{b,c}$	0.14
	18:3 + 20:1	0.01^{a}	0.04^{a}	1.59	0.20^{a}	0.05^{a}	0.04^{a}	0.09
	20:4	0.03^{b}	0.08^{b}	0.15^{b}	0.33^{a}	0.34^{a}	0.04^{b}	0.05
	22:1			3.44	1.35	0.82		0.16
60 days	12:0	0.02^{b}	0.48	0.05^{b}	0.04^{b}	0.30^{a}	0.29^{a}	0.03
	14:0	0.05^{b}	0.34	0.08^{b}	0.05^{b}	0.20^{a}	0.22^{a}	0.03
	16:0	0.50^{b}	0.87^{a}	$0.64^{a,b}$	0.51^{b}	0.56^{b}	$0.66^{a,b}$	0.79
	16:1	0.06^{c}	0.19^{a}	$0.16^{a,b}$	$0.09^{b,c}$	$0.09^{b,c}$	$0.07^{b,c}$	0.03
	18:0	$0.19^{a,b}$	0.16^{b}	$0.19^{a,b}$	0.16^{b}	0.11^{b}	0.26^{a}	0.03
	18:1	0.86^{a}	1.02^{a}	1.45	1.03^{a}	0.95^{a}	0.86^{a}	0.13
	18:2	0.82^{a}	0.10^{b}	0.70^{a}	0.68^{a}	0.18^{b}	0.87^{a}	0.13
	18:3 + 20:1	0.01^{b}	0.02^{b}	0.54	0.19^{a}	0.18^{a}	$0.07^{a,b}$	0.04
	20:4	0.03^{a}	0.05^{a}	0.11^{a}	0.11^{a}	0.05^{a}	0.09^{a}	0.03
	22:1			1.08	0.46^{a}	0.24^{a}		0.12

Results are expressed as mg of fatty acid/g heart.

SM_c, common standard error of the means; SO, sunflower oil; CO, coconut oil; RSO, rapeseed oil; SO-RSO, CO-RSO, SO-CO, 50/50 blends of respective oils.

$^{a-c}$In each line, treatments followed by the same letter are not significantly different ($P < 0.05$).

difference between RSO blend groups and diet groups without RSO. In groups fed diets containing RSO, the TG 18:2 content decreased with time and after 60 days CO-RSO TG 18:2 levels were many times lower than that observed in other groups where it was statistically the same. To sum up, when CO was blended into RSO, (a) TG 22:1 level was decreased more than when SO was blended, (b) TG 18:1 level was unaffected and (c) TG 18:2 level was reduced in comparison to the RSO group.

Heart PL fatty acid composition is shown in Table V. After 5 days, PL 18:1 level was significantly higher in CO-RSO and CO than in other diet groups. After 60 days, PL 18:1 level was increased in the CO group and was still significantly higher than that of other groups but the difference observed between CO-RSO and RSO after 5 days was no longer significant. Differences observed between the diet groups for PL 18:2, 20:4 and 22:6 levels were only significant after 60 days. After this period, PL 18:2 level was the lowest in CO and the same in CO-RSO, SO-RSO and SO groups where it was highest and significantly greater than in the RSO group. Thus, CO in RSO induced a higher PL 18:2 level than that observed when both these oils were fed separately. After 60 days, PL 20:4 level was the lowest in CO-RSO and highest in SO. Addition of CO to SO significantly lowered the 20:4 level in comparison to SO. A similar decrease also was found when CO was blended to RSO. Addition of SO to RSO, on the contrary, increased 20:4 in comparison to RSO; neither of these differences were significant and only CO-RSO and SO-RSO were significantly different. However, since these findings also were observed after 5 days, it is probable that the absence of significant differences was mainly a result of the large error encountered owing to the number of animals used.

After 60 days, the PL 22:6 level was highest in RSO and CO-RSO and significantly greater than that found in other diet groups, and was lowest in the SO group. Thus, the 22:6 level was unchanged by addition of CO to RSO in comparison to RSO, whereas it was lowered by addition of SO.

DISCUSSION

In these experiments, no synergism was observed between digestibility of coconut saturated fatty acids and rapeseed oil unsaturated fatty acids as has been reported in poultry between tallow long chain saturated fatty acids and rapeseed oil fatty acids (13-16). On the contrary, digestibility of saturated medium

TABLE V

Composition of Phospholipid Fatty Acids of Hearts of Rats Fed Either Undiluted or Blended Sunflower, Coconut and Rapeseed Oil As Dietary Oil

Dietary treatment		SO	CO	RSO	SO-RSO	CO-RSO	SO-CO	SM_c
	Fatty acids							
5 days	14:0	0.03^{b}	0.12^{a}	0.05^{b}	$0.07^{a,b}$	0.05^{b}	0.12^{a}	0.02
	16:0	1.27^{a}	1.58^{a}	1.28^{a}	1.27^{a}	1.58^{a}	1.34^{a}	0.13
	16:1	0.09^{a}	0.21	0.08^{a}	0.10^{a}	0.11^{a}	0.11^{a}	0.03
	18:0	3.14^{a}	2.06	2.33	3.12^{a}	2.44	2.57	0.02
	18:1	0.91^{b}	1.68^{a}	1.16^{b}	1.13^{b}	1.64^{a}	1.13^{b}	0.11
	18:2	3.13^{a}	2.48^{a}	2.51^{a}	3.29^{a}	2.92^{a}	3.41^{a}	0.29
	20:4	2.49^{a}	2.48^{a}	2.07^{a}	2.30^{a}	1.88^{a}	2.06^{a}	0.23
	22:1	–		0.22	0.22	Trace		0.06
	22:6	0.78^{a}	1.08^{a}	1.11^{a}	0.88^{a}	0.96^{a}	1.12^{a}	0.12
60 days	14:0	0.03^{b}	0.23^{a}	0.06^{b}	0.02^{b}	0.04^{b}	0.18^{a}	0.04
	16:0	$1.40^{b,c}$	1.83^{a}	1.32^{c}	1.33^{c}	1.79^{a}	$1.68^{a,b}$	0.11
	16:1	0.08^{a}	0.31	0.15^{a}	0.14^{a}	0.14^{a}	0.19^{a}	0.04
	18:0	3.68^{a}	2.99^{c}	2.91^{c}	$3.17^{b,c}$	2.84^{c}	$3.54^{b,c}$	0.15
	18:1	1.08^{c}	2.84	$1.49^{a,b}$	$1.24^{b,c}$	1.78^{a}	1.04^{c}	0.11
	18:2	3.60^{a}	1.90	2.74^{c}	3.48^{a}	$3.35^{a,b}$	$2.98^{b,c}$	0.15
	20:4	3.67^{a}	$3.30^{a,b}$	$3.08^{b,c}$	$3.40^{a,b}$	2.71^{c}	$2.88^{b,c}$	0.19
	22:1			0.22	0.11^{a}	0.13^{a}		0.03
	22:6	0.53^{c}	0.87^{b}	1.44^{a}	0.99^{b}	1.39^{a}	$0.77^{b,c}$	0.09

Results are expressed as mg/g fresh heart.

SM_c, common standard error of the means; SO, sunflower oil; CO, coconut oil; RSO, rapeseed oil; SO-RSO, CO-RSO, SO-CO, 50/50 blends of respective oils.

$^{a-c}$In each line, treatments followed by the same letter are not significantly different ($P < 0.05$).

For simplification, minor components such as 12:0, 18:3, 20:1 and 22:5 are not reported here.

chain fatty acids such as 12:0 was lowered when coconut oil was blended with rapeseed oil. Since the erucic acid digestibility also was lowered when rapeseed oil was mixed with another oil, it is tempting to suggest that competitive absorption or inhibition effects exist between fatty acids, especially since such phenomena are known for long chain unsaturated fatty acids (23,24) as well as between short and medium chain fatty acids and long chain fatty acids (25-27).

This work confirms the rare data obtained from the myocardium with adult rats (28) and shows that there is a less important triacylglycerol steatosis in adults than in weanling rats. However, while the fact that the triacylglycerol lipidosis was proportional to the dietary erucic acid content is not surprising, the observation that cardiac TG erucic acid level was dependant on dietary fatty acid composition is relatively unexpected. When saturated short and medium chain fatty acids of coconut oil were added to rapeseed oil, after 5 days, the TG 22:1 level was lower than with unsaturated long chain fatty acids of sunflower. Because hearts of rats fed the CO-RSO blend also had a total content of 18:1 (TG + PL) higher than hearts of rats fed other blends, it is possible that the CO-RSO blend enhanced erucic acid catabolism, as was recently observed by Rocquelin (12) after addition of linolenic acid to rapeseed oil. However, since 18:1 PL content was always highest in the CO group, it is possible that the high 18:1 level observed in the SO-RSO group does not result simply from an increase of erucic catabolism. Moreover, since the 12:0, 14:0 and 16:0 TG levels in CO-RSO were greater than those observed in SO-RSO, it is more likely that the decrease in the TG 22:1 level in CO-RSO compared to SO-RSO could be explained by a preferential uptake by heart of saturated medium chain fatty acids, compared to long chain fatty acids and particularly to erucic acid.

Addition of coconut oil to rapeseed oil did not significantly change polyunsaturated fatty acid levels in heart phospholipids compared to undiluted rapeseed oil, except for the 18:2 level which was enhanced. It is probable that this effect results from the synthesis of linoleic positional isomers induced by dietary coconut oil as shown by Leveillé et al. (29). Low and high cardiac contents of 20:4 and 22:6, respectively, in RSO or CO-RSO in comparison to SO and SO-RSO indicate that, while addition of sunflower oil reduced specific effects of dietary RSO on PL fatty acids, addition of medium chain fatty acids failed to. If the Beare-Rogers and Nera hypothesis (10) is correct, i.e., if the docosahexenoic acid level is a better indicator

of cardiac pathology than summations or ratios of polyenoic acids, the dilution by half of rapeseed oil by coconut oil probably should cause the same damage in rat myocardium as the consumption of a double dose of undiluted rapeseed oil. Thus, these results obtained on adult rats show analogies with data obtained in ducklings, animals that are particularly sensitive to effects of rapeseed oil, and where dietary rapeseed oil supplemented with trilaurin enhances lesions, mortality and decreases growth (8).

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Nitric-oxide Myoglobin As an Inhibitor of Lipid Oxidation[1]

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ABSTRACT

The effect of nitric-oxide myoglobin (MbNO) on lipid oxidation was studied in linoleate and β-carotene-linoleate aqueous model systems and compared with that of metmyoglobin (MMb) and oxymyoglobin (MbO_2) in short- and long-term reactions. While MMb and MbO_2 had a clear prooxidative effect, MbNO, under the same conditions, acted as an antioxidant. The specific antioxidative activity of MbNO was maintained even in the presence of prooxidants such as hemeproteins. The significance of the conversion of MbO_2 and MMb into an antioxidant during the curing process is discussed.

INTRODUCTION

Sodium nitrite, used to cure meat, produces products with microbiological stability, desirable color appearance and specific organoleptic characteristics.

Inhibition of "warmed over" flavors and lipid oxidation in cured meat by nitrite as measured by carbonyl compounds using gas chromatograpy (1-2), TBA value (3-4) and panelists' perceptions (5-6), was demonstrated, and reviewed by Pearson et al. (7).

Recently, it was reported that almost all the added nitrite was found as nitrosothiols and nitric-oxide myoglobin, and also in the form of protein-bound nitrite, nitrite, nitrate and gaseous nitrogen compounds (8-10). It is well known that nitric-oxide hemeproteins and predominantly nitric-oxide myoglobin represented the main color constituents of cooked cured meat (11-13).

Hemeprotein muscle pigments and nonheme iron have been implicated as prooxidants in meat lipid oxidation (14-18). While the prooxidant activity of hemes toward lipid oxidation has been known for many years, it has been recognized more recently that heme compounds also can act as antioxidants (19-23).

The purpose of this work was to study the role of nitric-oxide myoglobin on lipid oxidation in model systems in order to understand the stabilizing effect of nitrite on the lipid fraction in meat products, as well as its mechanism.

EXPERIMENTAL

Metmyoglobin (type I) (MMb) and β-carotene were purchased from Sigma Chemical Co., St. Louis, MO; sodium nitrite, ascorbic acid and butylated hydroxytoluene (BHT) from British Drug Houses Ltd., Poole, England; and linoleic acid was obtained from Fluka AG, Buchs, SG, Switzerland.

Nitric-oxide myoglobin was prepared according to the Fox and Thomson method (24). The reaction was carried out in a 0.1 M acetate buffer, pH 4.5, at 24 C under nitrogen atmosphere and in the dark.

The reaction was run by first mixing stock solution (5 ml) buffer metmyoglobin and ascorbic acid (10 ml); 3 min later, sodium nitrite and buffer were added, to a final volume of 25 ml. The concentration of the reactants in the final reaction mixture was myoglobin 0.5 mM, ascorbic acid 100 mM and sodium nitrite 50 mM.

After 15 min at 24 C, 3.5 ml of the reaction mixture was transferred to a column (30 cm × 2 cm) containing Sephadex G-25 (Farmacia Fine Chemicals AB, 20-80 μ). The elution was carried out with 0.1 M acetate buffer at pH 5.6. Four-ml fractions were collected and tested for hemeproteins at 400 nm. Ascorbic acid was determined by potentiometric titration with 2,6-dichloroindophenol (25).

Oxymyoglobin was prepared according to the Koizumi and Nonaka method (26) by reduction of metmyoglobin with $Na_2S_2O_2$ and subsequent passage over a column (25 cm × 2 cm) of Sephadex G25. The elution was carried out as already described for nitric-oxide myoglobin. The absorption spectrum of hemeproteins eluted from Sephadex G25 was characteristic of MbNO, MbO_2 and MMb.

The determination of MMb, nitric-oxide myoglobin (MgNO) and oxymyoglobin (MbO_2) concentrations after chromatographic elution for the antioxidative activity reaction was done by a spectrophotometer at 280 nm.

The assay of carotene-oxidizing activity was carried out according to a colorimetric method

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(27,28). Briefly, the technique consists of following the decrease in absorbance at 460 nm in the cuvette of a double-beam recording spectrophotometer. The test sample contained 1.5 ml buffered carotene-linoleate at pH 5.6, 0.1-0.4 ml of active fractions, and distilled water to a final vol of 2.0 ml. Concentrations in the initial reaction mixture were as follows: β-carotene, 1.4×10^{-5} M; linoleate, 2×10^{-3} M; Tween-20, 0.05%, buffer acetate, pH 5.6, 0.1 M. The blank sample contained all the reagents except β-carotene. The initial rate of decrease in absorbance was computed from a recorded graph and converted into the rate of carotene decrease in concentration (μM).

The assay of linoleate diene-conjugation was carried out by the Ben-Aziz et al. method (29). The reaction mixture contained linoleic acid, 4×10^{-4} M, and Tween-20, 0.05%, in a 0.1 M buffer acetate at pH 5.6. Both assay methods employed a DB Varian 634 recording spectrophotometer.

The coefficient of variation between triplicates were 4.2% (β-carotene destruction) and 5.7% (diene-conjugation).

RESULTS AND DISCUSSION

The activity of MbNO in lipid oxidation was studied and compared under the same experimental conditions and concentration as that of MMb and of MbO_2, since it is known that hemeproteins can act both as catalysts and inhibitors of lipid oxidation (21-23). The term "catalyst-inhibitor conversion" has been applied by Uri and his coworkers (30-31) to situations in which heavy metal catalysts at low concentrations become inhibitors at high concentration. Betts and Uri (30) explain the inversion effect shown by metal chelates by a relatively inefficient initiation reaction, first-order with respect to the chelate, which competes with a second-order termination reaction. Therefore, above a certain metal concentration, inhibition is bound to exceed initiation. It was later shown (31) that the chelates act as hydroperoxide decomposers (initiation), but at the same time react with the free radicals generated in the process (termination). More recently, we found that an AA-Cu^{2+} couple acts in a similar way (32). Their observations are parallel to those observed with unsaturated fats and heme compounds and the mechanisms may be related.

In the range of tested concentrations (2.2×10^{-6} M–2×10^{-5} M), MMb acted as a prooxidant toward carotene bleaching and linoleate oxidation, whereas MbNO acted as an antioxidant (Figs. 1 and 2). When compared with that of MMb, MbO_2 showed a somewhat stronger prooxidative activity (Fig. 3).

The effectiveness of MbNO as an antioxidant in long-term reactions was shown as well (Table I). It was found that if the concentration of

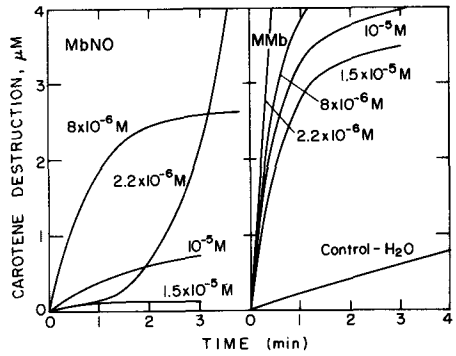

FIG. 1. Effect of nitric-oxide myoglobin (MbNO) and metmyoglobin (MMb) on carotene destruction.

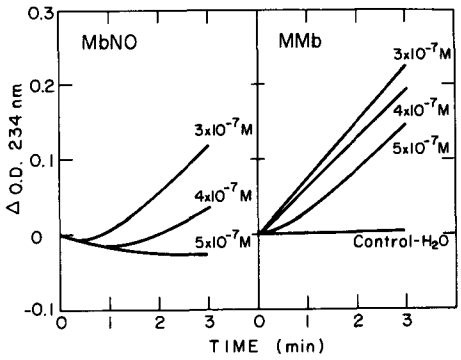

FIG. 2. Effect of nitric-oxide myoglobin (MbNO) and metmyoglobin (MMb) on linoleate diene-conjugation.

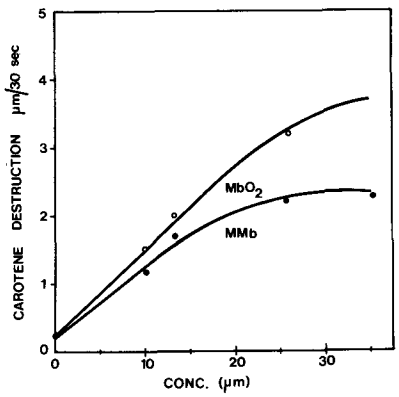

FIG. 3. Effect of metmyoglobin (MMb) and oxymyoglobin (MbO_2) on carotene destruction.

TABLE I

Carotene Destruction and Diene Conjugation in Long-term Reactions

Treatment	Carotene destruction[a] μM/20 min	Diene conjugation[b] od 234 nm/20 min
Control-H_2O	2.2	0.1
Metmyoglobin	4.2	1.2
Nitric-oxide myoglobin	0.1	-0.1

[a]Metmyoglobin and nitric-oxide myoglobin conc. = 1.3×10^{-5} M.
[b]Metmyoglobin and nitric-oxide myoglobin conc. = 2.1×10^{-6} M.

MbNO is above the critical antioxidant concentration, lipid oxidation was inhibited for a long time.

MbNO activity also was tested in the presence of heme-type prooxidants (Figs. 4 and 5) and lipoxygenase (in preparation). Its antioxidative activity was maintained even after these strong prooxidants were added, and it was highly effective at 10^{-5} M. As known from the literature and found also in our model system, a high concentration of MMb could inhibit lipid oxidation generated by prooxidants, whereas only a 5-8-fold concentration of MMb greater than MbNO provides an antioxidative effect (Fig. 4). Compared with BHT, MbNO has an almost similar antioxidant activity (Fig. 5).

It was proposed by several researchers (33-34) that nitrite forms a complex with iron porphyrin in heat-denatured meat, thus preventing its prooxidative effect. This inactivation was attributed to the valence state (+2) of the iron.

More recently, Koizumi et al. (35) and Koizumi and Nonaka (26) found that the rates of hemeprotein-catalyzed oxidation of lipids, are similar to the ferrous and ferric forms. Our results support this (see Fig. 3).

The stabilizing effect of MbNO may be attributed to the fact that Fe^{+2} is tied by nitric-oxide. However, this cannot explain the antioxidant effect of MbNO in the presence of prooxidants or in long-term reaction while the controls oxidized more rapidly than the samples with MbNO.

The mechanism proposed by us for the antioxidative effect of MbNO on lipid oxidation is based on the quenching effect of nitroxide radicals to free radicals involved in lipid autoxidation (36-39).

MbNO gave electron spin resonance spectra similar to organic nitroxide free radicals with an unpaired electron. The unpaired electron is associated with the NO group (40-41). The concept by which a nitroxide radical acts as an antioxidant by direct coupling with an alkyl

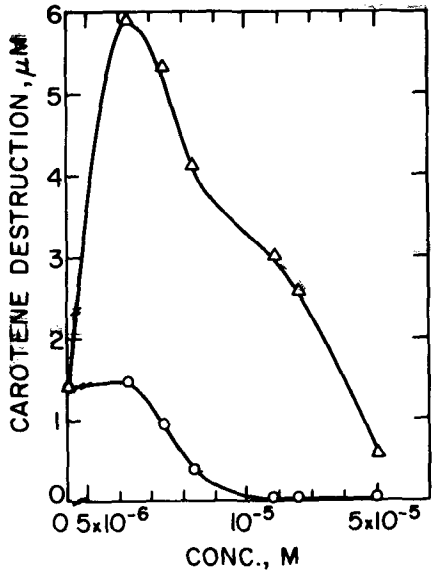

FIG. 4. The effect of nitric-oxide myoglobin (○) concentration and metmyoglobin (△) on the destruction of carotene by (2 μM) metmyoglobin as a prooxidant (data for the first minute of the reaction).

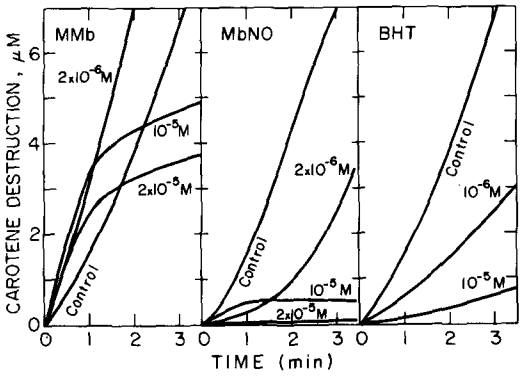

FIG. 5. The inhibitory effect of metmyoglobin (MMb), nitric-oxide myoglobin (MbNO) and BHT on carotene destruction by metmyoglobin (2 μM).

radical was first suggested by Thomas and Tolman (42) and Brownlie and Ingold (43). Weill et al. (37) showed that some stable synthetic nitroxides were quite effective antioxidants. More recently, it was shown that proline nitroxide and ethoxyquin, which are formed during lipid oxidation, have strong antioxidant activity (38-39).

We believe that MbNO acts in the early stages of the reaction to neutralize substrate-free radicals, and thus inhibits oxidation. During this reaction, the molecule of MbNO dissociates and MMb is left in the system. The concentrations of the free radicals and the hydroperoxides which are then left in the system dictates its subsequent behavior.

MbNO, at low concentration, resulted in a short time induction period (Figs. 1 and 4), whereas MMb, which remains in the system at a low concentration, acts as a prooxidant. However, if the concentration of MbNO is high enough, a rapid termination of the autoxidation reaction is achieved.

The rapid termination in lipid autoxidation achieved by MbNO in our model systems could be attributed both to (a) the MbNO- radical which quenches free radicals and thereby lowers the level of prooxidants in the system, and (b) MMb, which, derived in the model from MbNO, acts as a hydroperoxide decomposer and also a quencher of free radicals in a medium low in prooxidants (free radicals and hydroperoxides).

In light of these data, it is concluded that the stabilizing effect of nitrite on the lipid fraction in cured meat is derived from new compounds that are initiated during the process itself. One of those compounds is S-nitrosocysteine, which recently was found to act as an antioxidant in model systems and meat (44). Hemeproteins, which act as prooxidants in cooked meat, are inverted during the curing process to nitric-oxide hemeproteins with antioxidative activity. This inversion in hemeprotein's activity from a catalyst to an inhibitor of lipid oxidation seems to greatly improve the stability of cured meats.

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Fatty Acid and Sterol Composition of Ungerminated Spores of the Vesicular-Arbuscular Mycorrhizal Fungus, *Acaulospora laevis*

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ABSTRACT

The fatty acids and sterols of ungerminated chlamydospores of the vesicular-arbuscular (VA) endophyte *Acaulospora laevis* were examined by gas chromatography and mass spectrometry. The total lipid content of the spores was 45.5% of the spore dry weight. Predominant fatty acids were palmitoleic (52.5%), palmitic (25.5%) and oleic (7.4%). Minor fatty acids consisted of a range of (n-3) and (n-6) polyunsaturated acids. The occurrence of (n-3) polyunsaturated fatty acids is rare in fungi of the order Mucorales. Three sterols were identified as 24-ethylcholesterol (79.9%), cholesterol (11.0%) and 24-methylcholesterol (9.2%). No ergosterol was detected. Lipids of the chlamydospores of *A. laevis* are compared with those of *Glomus caledonius*.

INTRODUCTION

The biology of the vesicular-arbuscular (VA) mycorrhizal plant relationships has stimulated a lot of interest because of their potential importance in the uptake of nutrients by higher plants.

There have been few biochemical studies on the spores of VA endophytes. However, there have been many reports of lipid droplets in the spores and hyphae of the fungi (1-4). A study of the distribution, quantity and composition of the lipids in uninfected roots, of roots infected with *Glomus mosseae* and the external mycelium revealed that the VA endophyte-infected roots contained more total lipid than uninfected roots and that the mycelium had high levels of neutral lipids (2).

Although spores of *Acaulospora laevis* cannot be grown in axenic culture, spores can be grown in pot culture in sufficient quantity for chemical analysis. This paper reports the fatty acid and sterol composition of ungerminated spores of the VA endophyte *A. laevis*, and compares the results with those previously reported for the lipids of the VA endophyte *G. caledonius* (5,6).

EXPERIMENTAL PROCEDURES

A. laevis spores were grown on the roots of *Trifolium subterraneum*, in soil which had been steam-treated (7). The spores were separated from air-dried soil, as previously described (7). Spores were dried in vacuo over KOH pellets for 24 hr prior to weighing. Immediately after separation, 600-1,000 *A. laevis* spores were disrupted in a screw-capped glass mortar and pestle, containing a small vol of methanol at 0 C. The preparation was checked microscopically to ensure that all spores were fractured. The disrupted spores were then extracted by shaking on a wrist shaker for 1 hr in 2 ml chloroform/methanol (2:1) at 0 C. This extraction process was repeated twice and the third extraction was done at 40 C. The chloroform/methanol extracts were washed with 0.2 vol of saline (0.9% w/v NaCl), and the aqueous phase was extracted by the Folch et al. method (8) as previously reported (5). The pooled chloroform phases were concentrated under nitrogen.

Fatty acids were analyzed as their methyl esters, which were prepared by transesterification of the neutral lipid extract using methanol containing 3% H_2SO_4 (9). Free sterols and sterol esters were isolated by 2-dimensional thin layer chromatography (TLC) (10). Sterol esters were hydrolyzed (9) and were acetylated together with the free sterols in pyridine/acetic anhydride (2:1) overnight at room temperature. The residue of the chloroform/methanol-extracted spores was hydrolyzed by refluxing with 20% aqueous KOH for 2 hr. Bound sterols were extracted into petroleum ether (bp 30-60 C) and bound fatty acids were recovered in petroleum ether after acidification with 6 N HCl (9). Both sterols and fatty acids were derivatized as before.

Fatty acids and sterols were analyzed by gas liquid chromatography (GLC), using a Varian Aerograph 2700 gas chromatograph equipped with a flame-ionization detector. Fatty acid methyl esters were separated on 1.8 m x 3.4 mm glass columns packed with 10% EGSS-X on Supelcoport 100/120 mesh at 200 C and with 10% BDS (butanediol succinate polyester) on Supelcoport 100/120 mesh at 170 C. Fatty acid analysis was carried out isothermally; the injector and detector temperatures for all GLC

analyses were 270 C. Chain length and the degree of unsaturation of the fatty acids were verified before and after hydrogenation in methanol with platinum catalyst (9) by comparison of their relative retention times with authentic samples, and/or by graphic determination of equivalent-chain-length (ECL) values (11). Sterols were identified by comparison of their relative retention times with authentic samples (12). The amounts of individual sterols and fatty acid methyl esters were determined by comparison of peak areas to the internal standards, stigmasterol and methyl heptadecanoate, respectively. Peak area was determined by triangulation. The identity of major fatty acids and sterols was confirmed by mass spectroscopy (MS) with a Varian Matt 311 mass spectrometer, using an ionization potential of 70 eV. Results expressed are the means of 3 batches of spores grown in pot culture.

RESULTS AND DISCUSSION

The total lipid content of *A. laevis* chlamydospores was 45.5% of the spore weight, which is high compared to most fungal spores (13). *G. caledonius*, the only other VA endophyte spore which has been studied, displayed a similar lipid content of 45.5%, and this increased during germination to 70% of the spore weight (5). From the small amount of work that has been done on fungal spore lipids from the order Mucorales, it is apparent that they generally have lower lipid contents than either *A. laevis* or *G. caledonius* (13). High total lipid levels have been observed in rust spores, such as aeciospores of *Cronartium ribicola* and basidiospores of *C. fusiforme* with 18 and 31%, respectively (14,15).

The fatty acids of *A. laevis* spores were similar to those of other fungi and plants in that a large proportion of the total fatty acids, 90.6% were made up of 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3. However, the fatty acids of *A. laevis* and *G. caledonius* apparently are distinguished from most other fungi by the presence of a range of polyunsaturated fatty acids (5). *A. laevis* contains 9.4% of its total fatty acids as polyunsaturates (Table I). The C_{20}-polyenoic fatty acids comprise 8.5% of the total fatty acids, whereas the longer chain length fatty acids made up the remaining 0.7%. There were 2 odd-chain-length fatty acids identified, 23:3(n-6) and 25:3(n-6). However, they were present only in trace amounts. Fatty acids, as methyl esters, were identified by comparison of the retention times with those of authentic standards and also with fatty acid methyl esters previously identified by GLC-MS

TABLE I

Fatty Acids of *Acaulospora laevis*

Fatty acids	Neutral lipid fatty acids (%)	Bound fatty acids (%)
14:0[a]	0.6 ± 0.2[b]	
16:0	25.5 ± 0.5	2.3 ± 0.4
16:1(n-7)	52.5 ± 1.0	23.4 ± 0.6
18:0	0.9 ± 0.3	14.8 ± 0.9
18:1(n-9)	7.4 ± 0.3	25.3 ± 1.1
18:2(n-6)	1.7 ± 0.2	10.5 ± 0.8
18:3(n-6)	0.3 ± 0.1	6.9 ± 0.4
18:3(n-3)	0.3 ± 0.2	6.3 ± 0.5
20:2(n-6)	2.3 ± 0.4	3.9 ± 0.4
20:3(n-6)	2.0 ± 0.3	0.7 ± 0.2
20:3(n-3)	1.5 ± 0.2	5.9 ± 0.6
20:4(n-6)	1.1 ± 0.2	
20:5(n-3)	1.4 ± 0.3	
22:4(n-6)	0.3 ± 0.2	
22:5(n-6)	0.2 ± 0.1	
23:3(n-6)	t[c]	
24:1(n-9)	0.2 ± 0.1	
25:3(n-6)	t	
26:3(n-6)	t	

[a]Number of carbon atoms in acid:number of double bonds; n represents the number of carbon atoms between the terminal double bond and the methyl end of the molecule. Double bond position provisionally identified only.

[b]Relative percentage of fatty acids ± SD, from 3 batches of spores.

[c]t = trace, < 0.1%.

prepared from *G. caledonius* spores (5). Also, minor polyunsaturated fatty acids were identified by ECL values, before and after hydrogenation. The fatty acids identified in *A. laevis* are very similar to those identified in *G. caledonius*. However, the range of polyunsaturated acids was greater in *A. laevis*. The predominant fatty acids of *A. laevis* and *G. caledonius* were 16:1 52.9% and 47.7%, 16:0 25.6% and 26.0%, 18:1 7.7% and 15.4%, and 18:2(n-6) 1.9% and 2.5%, respectively. The only difference seen in the fatty acids identified in both spores was that *A. laevis* contained 20:4(n-6) whereas *G. caledonius* contained 20:4(n-3).

The major fatty acids of diatoms have been shown to be 16:1 and C_{20}-polyenoic acids (16); the presence of the polyunsaturated fatty acids 20:4 and 20:5 in the nonphotosynthetic diatom *Nitzschia alba* was characteristic of photosynthetic diatoms (17). Such acids may be involved in the function of the photosynthetic apparatus in algae (17). Similar inferences may be drawn for *A. laevis* and *G. caledonius*, i.e., perhaps these fungi once had photosynthetic capabilities but have since lost them. However, on the other hand, the poly-

TABLE II

Sterols of *Acaulospora laevis*

Sterols	Total sterols[a] (μg sterols/mg dry weight)	Free sterols (%)[b]	Sterol esters (%)	Bound sterols (%)
Cholesterol	0.18 ± 0.02	20.0 ± 2.4	0.5 ± 0.1	3.4 ± 0.5
24-Methyl-cholesterol	0.15 ± 0.03	10.3 ± 1.6	10.4 ± 1.2	1.5 ± 0.3
24-Ethyl-cholesterol	1.31 ± 0.2	69.6 ± 9.2	89.1 ± 12.3	95.1 ± 15.0

[a]The figures are the average μg sterol/mg dry weight of relative peak area ± SD obtained from 3 batches of spores.

[b]The figures are the average percentages of relative peak area ± SD obtained from 3 batches of spores.

unsaturated fatty acids may be membrane components. Polyunsaturated fatty acids guarantee high flexibility of membranes at low temperatures and are less susceptible to photooxidation at high daytime temperatures (18). Because of the ubiquitous nature of VA endophytes, polyunsaturated fatty acids may play an important role in helping maintain the viability of these organisms.

Bound fatty acid in *A. laevis* represented 1.2% of the total fatty acid and their range was less than that of the total fatty acids (Table I). Also, the distribution was quite different: 18:1(n-9), 16:1(n-7), 18:0 and 18:2(n-6) were the major fatty acids.

Sterols were extracted from spores of *A. laevis*, fractionated by TLC, acetylated and separated by GLC, using a 3% SE-30 column (1.8 m × 3.4 mm). Tentative identifications based on retention data of the 3 sterols resolved were cholesterol, 24-methylcholesterol and 24-ethylcholesterol (Table II) (12). The major sterol that was separated represented 79.9% of the total sterols in the ungerminated spores and gave a GLC retention time that corresponded to 24-ethylcholesterol (12). The mass spectrum of this sterol acetate showed ion peaks at m/e 396 [M^+-(acetate)], 381 [M^+-(CH_3 + acetate)], 255 [M^+-(side chain + acetate)] and 213 [M^+-(side chain + 42 + acetate)], indicating a monounsaturated C_{29} sterol acetate with the double bond in the steroid nucleus, thereby confirming the structure to be 24-ethylcholesterol.

The second most abundant sterol acetate represented 11.0% of the total sterols and had a GLC retention time that corresponded to cholesterol acetate (12). The mass spectrum showed ion peaks at m/e 368 [M^+-(acetate)], 353 [M^+-(CH_3 + acetate)], 255 [M^+-(side chain + acetate)] and 247 [M^+-(acetate + C_9H_{13})], confirming the structure of this sterol acetate to be that of cholesterol. The smallest sterol component isolated comprised 9.2% of the total sterols and had a GLC retention time that corresponded to 24-methylcholesterol (12). The mass spectrum of this sterol acetate showed ion peaks at m/e 382 [M^+-(acetate)], 367 [M^+-(CH_3 + acetate)] 255 [M^+-(side chain + acetate)] and 213 [M^+-(side chain + 42 + acetate)], indicating a monounsaturated C_{28} sterol acetate with one double bond in the steroid nucleus, thus confirming the structure to be 24-methylcholesterol.

The total sterol content for spores of *A. laevis* was 0.16% of the spore weight. This is lower than the 0.39% sterol content reported for *G. caledonius* (6), but is still higher than the range reported for some fungi in the order Mucorales, which ranged from undetectable to 0.025% of mycelium dry weight (19). The total sterol content of *A. laevis* consisted of 49.8% free sterols, 34.4% sterol esters and 15.8% bound sterols. The major sterol in all fractions studied was 24-ethylcholesterol (Table II), followed by cholesterol in the free and bound sterols, and 24-methylcholesterol in the sterol esters. Ergosterol is considered to be the major fungal sterol, and has been more frequently reported in the Mucorales than has cholesterol (13). However, no ergosterol was detected in *A. laevis*. The lipids in spores of *G. caledonius* and *A. laevis* have similar total lipid contents: both contain a large range of fatty acids with (n-3) and (n-6) polyunsaturated fatty acids, and cholesterol, 24-methylcholesterol and 24-ethylcholesterol.

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Altered Fatty Acid Desaturation and Microsomal Fatty Acid Composition in the Streptozotocin Diabetic Rat

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ABSTRACT

Streptozotocin diabetes in the rat diminishes the synthesis of both monounsaturated and polyunsaturated fatty acids. Rat liver microsomal fatty acid composition and fatty acid desaturation were studied in the streptozotocin diabetic rat. The major alterations in fatty acid composition found in the diabetic rat were decreased proportions of palmitoleic, oleic and arachidonic acids and an increased proportion of linoleic and docosahexaeneoic acids. These findings, other than the increased docosahexaeneoic acid, probably result from the diminished liver microsomal Δ9 and Δ6 desaturase activities found in these animals. These changes are not due to the diminished weight gain of the diabetic animals since restricting food intake of control animals to achieve a similar weight gain failed to reproduce either the changes in fatty acid composition or the decrease in fatty acid desaturation. The increased food intake of the diabetic animal may contribute to the altered proportions of linoleic and arachidonic acids since limiting food intake in diabetic animals to that of normal controls diminished the magnitude of these changes. Insulin therapy for 2 days not only reverses and overcorrects the diminished desaturase activities, but likewise reverses and overcorrects the altered fatty acid composition, with the exception of the diminished arachidonic acid levels which are further decreased following insulin therapy. These findings strongly suggest that most of the changes in fatty acid composition in the diabetic rat are indeed caused by the diminished fatty acid desaturase activities.

INTRODUCTION

Diminished microsomal fatty acid desaturation occurs in experimental diabetes mellitus and is correctable with 24-hr insulin therapy (1-3). The Δ9, Δ6 and Δ5 desaturase enzyme activities are all diminished in diabetes even though these probably are different enzymes (4). The Δ9 desaturase enzyme converts saturated fatty acids into monounsaturated fatty acids. The Δ6 desaturase enzyme converts 9,12-18:2 (linoleic acid) to 6,9,12-18:3 (γ-linolenic acid), the initial and rate-controlling step in the conversion of linoleic to 5,8,11,14-20:4 (arachidonic acid) (5). The defect in experimental diabetes results from an effect on the terminal desaturase enzyme rather than an effect on the prior components of the microsomal electron transport system responsible for fatty acid desaturation (2,3). The principal physiological fatty acids whose synthesis is dependent on these enzymes are 9-16:1 (palmitoleic acid), 9-18:1 (oleic acid), 8,11,14-20:3 (eicosatrienoic acid), arachidonic acid and 4,7,10,13,16,19-22:6 (docosahexaenoic acid). These fatty acids also can be supplied by diet along with linoleic acid and 9,12,15-18:3 (linolenic acid), polyunsaturated fatty acid precursors which cannot be synthesized and can only be supplied by diet.

Previous studies have been carried out to determine if this diminished fatty acid desaturation results in altered fatty acid composition of lipids from liver, testis and adipose tissue (6-10). Although the findings have not been uniform, it has been generally concluded that alterations in fatty acid composition of certain lipids may be at least partly due to the impairment of fatty acid desaturation. The interpretation of these experiments has been made difficult by the presence of factors other than diabetes that may alter lipid fatty acid composition and by the failure to study specific subcellular components whose compositions vary considerably (11). Adipose tissue triacylglycerols reflect the fatty acid composition of the diet (12), as do serum and tissue phospholipids and cholesterol esters, although to a lesser extent. In an attempt to accentuate the changes in fatty acid composition in diabetes, some of the previous studies have been carried out in animals fed diets deficient in essential fatty acids (7,8,10), which normally accelerate fatty acid desaturation (4).

The purpose of this study was to determine if changes in liver microsomal fatty acid composition in experimental diabetes mellitus correspond to changes in fatty acid desaturation and if such changes are readily reversed by insulin therapy. Since the synthesis of unsaturated fatty acids occurs in the microsomal fraction, microsomal fatty acid composition might reflect early changes in fatty acid desaturation. We also have carried out studies to determine if

the increased food intake and/or lesser weight gain of the diabetic animals might contribute to observed changes in lipid desaturation and composition.

MATERIALS AND METHODS

Materials

[^{14}C] stearoyl-CoA and [1-^{14}C] linoleic acid were purchased from New England Nuclear. Unlabeled stearoyl-CoA was purchased from P&L Biochemicals. Linoleic acid, free coenzyme A, NADH and ATP were purchased from Applied Science Labs or Sigma Chemical. Streptozotocin was kindly supplied by the Upjohn Co. and the National Cancer Institute. All other chemicals were of reagent grade.

Animals and Their Treatment

White, male, Sprague-Dawley rats from Charles River Laboratories were maintained on a Purina Laboratory Chow #5001 diet ad libitum unless indicated otherwise. A sample analysis from one batch of the diet contained 5.0% total lipid with a fatty acid composition of 1.7% 14:0 (myristic acid), 22.9% 16:0 (palmitic acid), 1.9% palmitoleic acid, 7.8% 18:0 (stearic acid), 32.4% oleic, 29.8% linoleic and 3.1% linolenic acids. Experimental diabetes was produced by the intravenous injection of streptozotocin in a dose of 75 mg/kg body weight. After several days, blood glucose was estimated using Dextrostix reagent strips and an Ames Reflectance Meter. Only those rats with blood glucoses greater than 300 mg/dl were considered diabetic. Animals treated with insulin were given 4 U NPH insulin at 8 am and 4 pm for 2 days prior to death. Control and untreated diabetic animals were injected with saline. Before injection of insulin or saline and immediately before death, blood was taken for glucose determination by a commercial glucose oxidase method. Rats were killed 14-21 days after streptozotocin injection in the fed state, and liver microsomes were isolated.

Preparation of Microsomes

Microsomes were prepared from rats killed by a blow to the head followed immediately by cervical disarticulation. The liver was rinsed in 0.25 M sucrose, and was homogenized in 5 ml of 0.25 M sucrose/g liver. The homogenate was centrifuged for 20 min at 16,000 G, and the resulting supernatant was centrifuged for 60 min at 105,000 G. The surface of the microsomal pellet was washed 3 times with cold 0.25 M sucrose, and the pellet was resuspended in same to an appropriate protein concentration. In some instances, pellets were frozen for several days prior to use. All microsomal preparation was done at 0 to 4 C. When frozen in concentrated form (15 mg microsomal protein/ml 0.25 M sucrose), enzyme activities were stable for up to 2 weeks. Washed microsomes for lipid and fatty acid analysis were prepared by resuspending the 105,000 G pellet in 0.25 M sucrose and centrifuging for 60 min at 105,000 G.

Enzyme Assays

The desaturation reactions were carried out as described previously (2). Stearoyl-CoA desaturation reaction mixtures containing 36 μM [1-^{14}C] stearoyl-CoA (200,000 dpm), 1 mM NADH, 50 mM potassium phosphate buffer (pH 7.4) and ca. 1 mg microsomal protein in a total vol of 1 ml were incubated at 37 C for 20 min. Linoleic acid desaturation reaction mixtures containing 40 μM [1-^{14}C] linoleic acid, 5 mM $MgCl_2$, 2 mM ATP, 50 μM coenzyme A, 1 mM NADH, 50 mM potassium phosphate buffer (pH 7.4) and ca. 2.5 mg microsomal protein in a total vol of 1 ml were incubated at 37 C for 15 min. Rates of desaturation are nearly linear with time under these conditions. The reactions were terminated by the addition of 2 ml of ethanolic KOH (25% 10 M KOH and 75% ethanol). The reaction mixtures were hydrolyzed at 70 C for 1 hr and titrated to a pH of 3-4 using bromophenol blue as indicator. The fatty acids were extracted with petroleum ether and methylated using boron trifluoride in methanol as described previously (13). The fatty acid methyl esters were separated by argentation thin layer chromatography (TLC) using a hexane/benzene (50:50) solvent system for the stearoyl-CoA desaturase assays and a toluene/acetone (95:5) solvent systems for the linoleic acid desaturase assays (2). ^{14}C incorporation into product was determined by the substrate and the product in a liquid scintillation spectrometer.

Lipid Fatty Acid Composition

Total microsomal lipid was extracted with chloroform/methanol (2:1) (14). Fatty acid methyl esters of the lipid extract were formed as just described following alkaline hydrolysis. Separation of individual fatty acid methyl esters was accomplished by gas liquid chromatography (GLC) using a Hewlett Packard 5711 gas chromatograph. Six-ft x 1/4 in. glass columns packed with 12% EGSS-X and 2% polyvinylpyrrolidinone on 100/120 mesh Chromosorb W (acid-washed) were used. The columns were operated isothermally for 45-75 min at 185 C using 60 ml/min nitrogen (carrier gas)

flow rate. In the initial studies reported herein, chromatography was carried out for 45 min/sample; consequently, the docosahexaenoate peak was excluded. Subsequently, chromatography was carried out for 90 min to include this peak. Only the fatty acids which were present in greater than 1% proportions are reported. Peaks were identified by comparison of retention times with authentic standards. The percentage of each fatty acid present was determined from the peak areas using a Hewlett Packard 5530 recorder-integrator. Analysis of a quantitative standard containing 20% by weight each of the methyl esters of palmitic, stearic, oleic, linoleic and linolenic acids revealed integrated areas between 19.88% and 20.18% for each component.

Analytical Procedures

Phospholipid, cholesterol and triacylglycerol assays were carried out on the chloroform/methanol extracts by previously described procedures (15-17). Proteins were determined by a modification of the Lowry method using bovine serum albumin as standard (18).

Statistical Analysis

The results of replicate experiments were pooled for statistical analysis. Significant differences between groups were determined using the one-way analysis of variance.

RESULTS

Weight Gain and Food Intake in Diabetic Animals

In the first series of experiments, the animals were divided into 4 groups. Group I was control animals and group II was diabetic animals fed ad lib. throughout the experiment. Group III was control animals whose food intake was restricted so that their weight gain would match the weight gain of the diabetics. Because diabetic animals have hyperphagia but gain less weight than controls, these food restricted controls were studied to determine if changes in desaturation or lipid composition in diabetes might be caused by the decreased weight gain and relatively increased fat mobilization of the diabetic animals. Group IV was diabetic animals whose food intake was restricted to match the food intake of the controls. These food-restricted diabetics were studied to determine if changes in desaturation or lipid composition in diabetes might be caused by the increased food intake of the diabetic animals.

Figure 1 illustrates the body weight and food intake of the 4 groups of rats throughout the experiment. At the end of the experiment, the diabetic rats (group II) had 24% greater food intake but a 55% lesser weight gain than the controls. The food-restricted controls (group III) received 68% of the food intake of

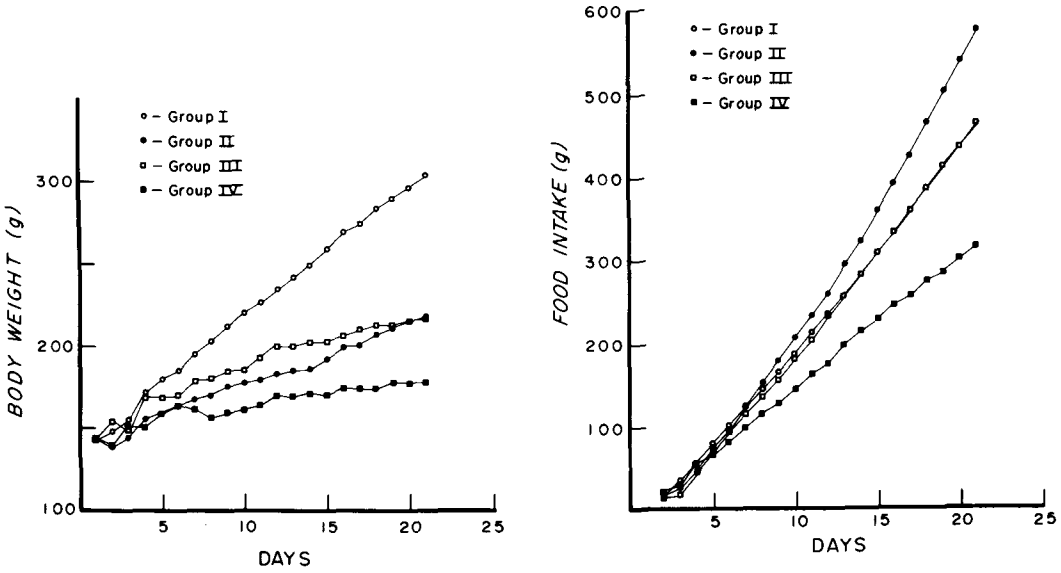

FIG. 1. Body weight and food intake in diabetes. Group I–controls fed ad lib. (n = 10); group II–diabetics fed ad lib. (n = 12); group III–controls whose food intake was restricted so that weight gain would match that in diabetics (n = 10); group IV–diabetics whose food intake was restricted to match food intake of controls (n = 10). Diabetes was produced and experiment carried out as described in Materials and Methods.

the controls in order to achieve a similar weight gain as the diabetics. The food-restricted diabetics (group IV) which were given the same food intake as the controls had an 80% lesser weight gain than control animals.

Influence of Food Intake and Weight Gain on Fatty Acid Desaturation in Diabetes

Figure 2 shows the results of the fatty acid desaturase activities in the 4 groups of rats. The diabetic rats had a 90% inhibition of stearate (Δ9) desaturation and a 48% inhibition of linoleate (Δ6) desaturation. Although the inhibition of Δ6 desaturation is not statistically significant in this small group of animals, combined results from the experiments in Figures 2 and 5 reveal a highly significant 34 ± 3% (mean ± SE, n = 11) inhibition of Δ6 desaturation in the diabetic animals. This is consistent with previous reports by ourselves and others (2,19). Control animals whose food intake was restricted so that their weight gain was equivalent to the diabetic animals had a marked increase in Δ6 desaturase activity to 314% of control and a lesser increase in Δ9 desaturase activity to 136% of control. These increases in fatty acid desaturase activities in the food-restricted controls were unexpected since fasting depresses both Δ9 and Δ6 desaturase activities (4) and emphasizes the need for evaluation of food intake as one important factor in the complex control of fatty acid desaturation. The diabetic animals whose food intake was restricted to match the food intake of the controls demonstrated decreases in desaturase activity similar to the diabetics fed ad lib.

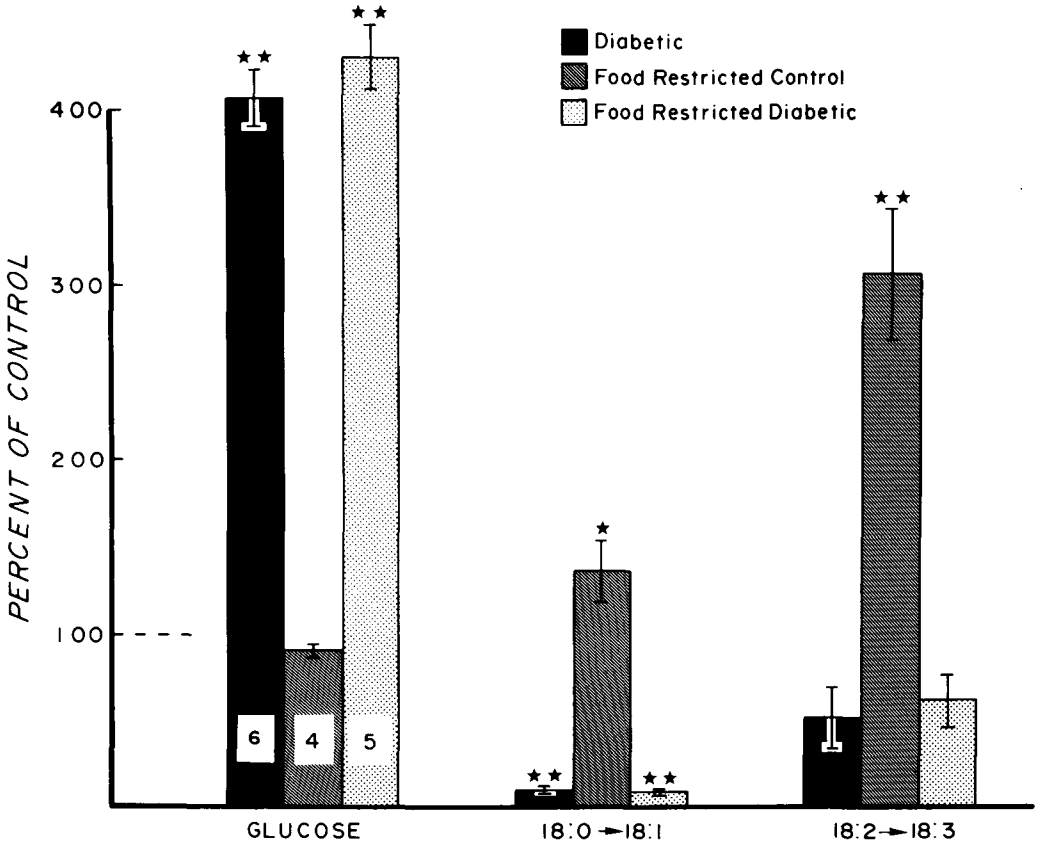

FIG. 2. Microsomal fatty acid desaturase activity in diabetes and with food restriction. Desaturase enzyme assays were carried out as described in Materials and Methods in the 4 groups of animals described in the legend to Fig. 1. Mean ± SEM control stearate (18:0→18:1) and linoleate (18:2→18:3) desaturase activities were 3.68 ± 0.30 and 0.92 ± 0.03 nmol/mg protein/incubation period, respectively. Mean ± SEM control blood glucose was 100 ± 2 mg/dl. The numbers of animals in each group are shown in the first set of bars with 4 animals in the control group. The details of the animal groups are given in the legend to Fig. 1. *Significantly different from controls, $p < .05$; **$p < .01$.

Microsomal Lipid Composition in Diabetes

Figure 3 shows the microsomal composition of the major lipid classes in the fed and food-restricted control and diabetic rats. Control liver microsomal lipids contained 85% phospholipid, 9% cholesterol and 5% triacylglycerol. The diabetics fed ad lib. had slightly higher phospholipids than controls. The food-restricted controls and diabetics both had slightly higher cholesterol contents than the controls. Because there were no major differences in composition of the major lipid classes in any of the groups, changes in fatty acid composition between the various treatment groups are likely caused by changes in fatty acid composition of the phospholipid fraction.

Microsomal Fatty Acid Composition in Diabetes

Figure 4 shows the results of the fatty acid analysis of the microsomal lipid extracts. Liver microsomal lipid fatty acid composition was altered in the diabetic animals fed ad lib. with significantly decreased proportions of palmitoleic, oleic and arachidonic acids and an increased proportion of linoleic acid. The decreased palmitoleic acid may be a result of the diminished Δ9 desaturase activity and the increased linoleic acid with decreased arachidonic acid may result from the diminished Δ6 desaturase activity found in these animals. Thus, these findings are consistent with the hypothesis that the altered lipid composition may result from diminished Δ6 and Δ9 fatty acid desaturation.

The food-restricted controls (group III) had significantly increased proportions of palmitoleic and oleic acids and a lower proportion of stearic acid than the controls fed ad lib. This may be a result of the increased Δ9 desaturation in these animals. No changes in proportions of linoleic or arachidonic acids were found, even though Δ6 desaturase activity was markedly increased in this group. Because none of the fatty acid composition changes in group III mimicked the changes in the diabetics, the major differences between the diabetics and controls fed ad lib. were not simply due to the lesser weight gain of the diabetics.

The fatty acid composition changes in the food-restricted diabetic animals (group IV) were similar to those in the diabetics fed ad lib. except that the increased linoleic acid and decreased arachidonic acid were of lesser magnitude, and the change in arachidonic acid was no longer statistically significant. In addition, the slight increase in stearic acid seen in the diabetics fed ad lib. was greater in the

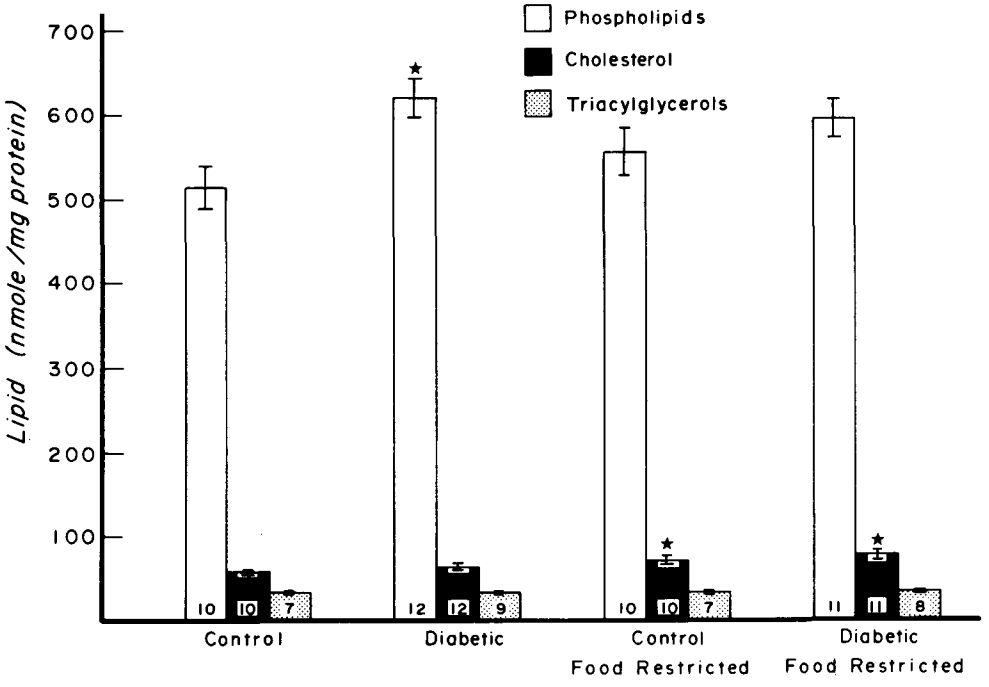

FIG. 3. Microsomal lipid composition in diabetes and with food restriction. The number of animals in each group is shown in the bars. Animal treatments are described in Materials and Methods. Results are shown as mean ± SEM. The asterisk indicates a significant difference from the controls, $p < .01$.

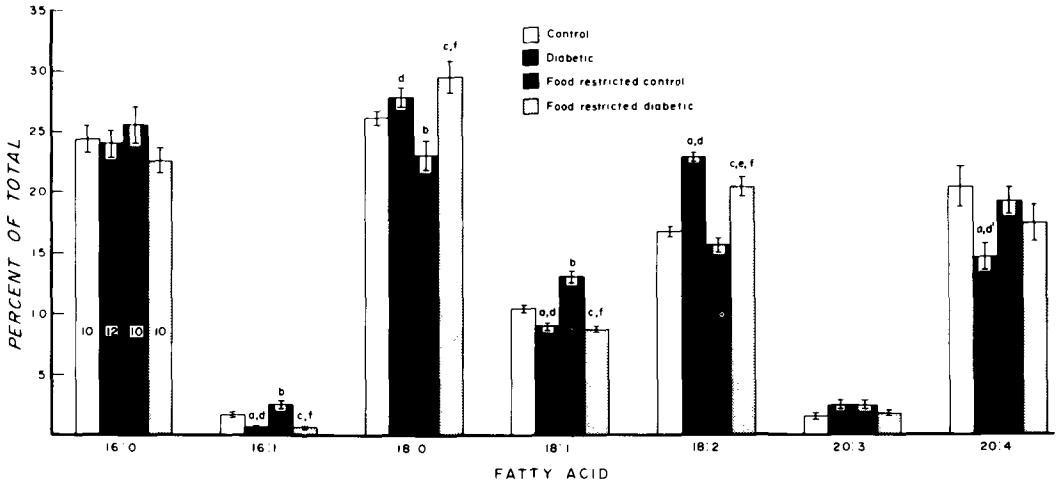

FIG. 4. Microsomal fatty acid composition in diabetes and with food restriction. Fatty acid composition was determined by gas chromatography as described in Materials and Methods in the groups of animals described in the legend to Fig. 1. The 20:3 fraction is 8,11,14-20:3. The number of animals in each group is shown in the first set of bars. Results are shown as mean ± SEM. The letters (x) above the bar indicate significant differences, $p < .01$, except x′ indicates $p < .05$. (a) Group I vs group II; (b) I vs III; (c) I vs IV; (d) II vs III; (e) II vs IV; (f) III vs IV.

food-restricted diabetics, and reached statistical significance. This suggests that the increased food intake of the diabetic animals cannot account for the changes in palmitoleic, stearic and oleic acids since these changes were the same or greater. However, the increased food intake may partially account for the changes in linoleic and arachidonic acids seen in the diabetic animals since food restriction limited the magnitude of these changes.

Fatty Acid Desaturase Activity in Insulin-Treated Diabetic Animals

In the next group of experiments, control, diabetic and diabetic animals that had been treated with 4 U NPH insulin twice a day for 2 days were studied. Figure 5 demonstrates that streptozotocin diabetes caused a 91% inhibition of stearate ($\Delta 9$) desaturation and a smaller but significant 26% inhibition of linoleate ($\Delta 6$) desaturation as already noted. Treatment of the diabetic animals with 4 U NPH insulin twice a day for 2 days lowered the blood glucose at time of sacrifice to 211 mg/dl from a pre-treatment mean of 403 mg/dl while causing not only repaired fatty acid desaturation, but a stimulation of $\Delta 6$ and $\Delta 9$ desaturase activities to values higher than control values. The insulin-treated animals had $\Delta 9$ desaturation rates of 479% of control and $\Delta 6$ desaturation rates of 172% of control. These findings are consistent with our previously published data (2).

Microsomal Fatty Acid Composition in Insulin-Treated Diabetic Animals

Figure 6 shows the fatty acid composition of liver microsomal lipid in the same control, diabetic and insulin-treated diabetic animals. The fatty acid composition in the diabetic animals was altered as before, with significantly decreased proportions of palmitoleic and arachidonic acids and increased proportions of linoleic acid. The decrease in oleic acid in these experiments was not statistically significant. In addition, in these experiments, there was a slight but significant decrease in palmitic acid and an increased proportion of docosahexaenoic acid, the level of which was not evaluated in the first series of experiments. The increased docosahexaenoic acid, a product derived from the desaturation and elongation of linolenic acid, may be related to diminished utilization of this fatty acid.

Also shown in Figure 6 is the effect of 2 days of insulin therapy on the fatty acid composition changes of the microsomal lipid. The increased docosahexaenoic acid level of the untreated diabetics was returned to normal. The decreased palmitic and palmitoleic acid and the increased linoleic acid levels were not only returned to normal but actually significantly changed in the opposite direction. Particularly striking was the increase in palmitoleic acid from 0.74% of total in the untreated diabetics to 6.5% in the insulin-treated diabetics, compared to a value of 1.9% in control animals.

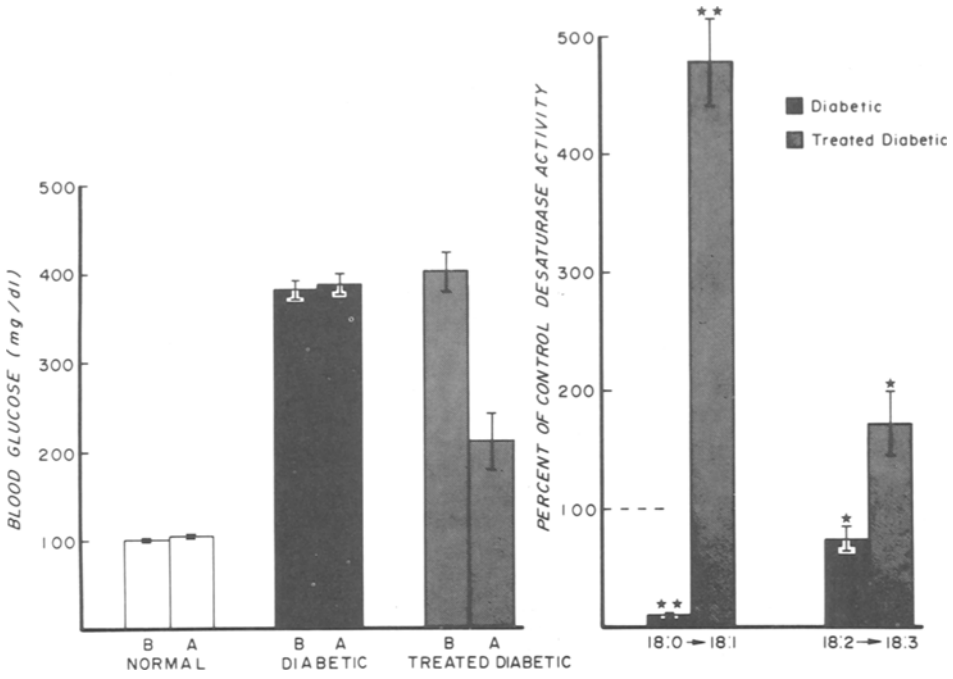

FIG. 5. Blood glucose and microsomal desaturase activities in insulin-treated diabetes. Desaturase enzyme assays and experimental design are described in Materials and Methods. There were 8 animals in each group, normal controls, untreated diabetics and insulin-treated diabetics. Mean ± SEM control stearate (18:0→18:1) and linoleate (18:2→18:3) desaturase activities were 4.18 ± 0.52 and 1.07 ± 0.11 nmol/mg protein/incubation period, respectively. *Significantly different from controls; $p < .05$; **$p < .01$. (A) At time of sacrifice; (B) before insulin or saline. Treated diabetics had significant reduction in blood glucose, $p < .01$.

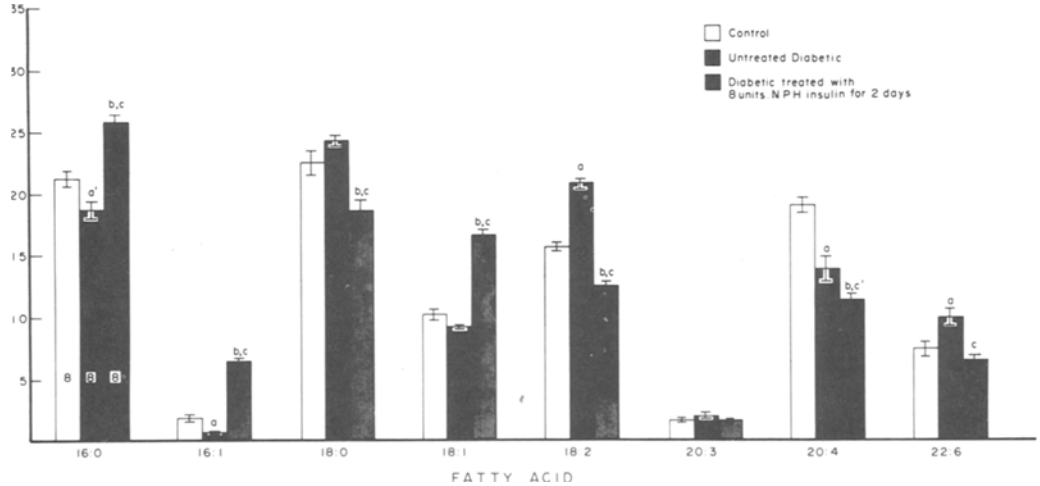

FIG. 6. Microsomal fatty acid composition in insulin-treated diabetics. Fatty acid composition was determined by gas chromatography as described in Materials and Methodsin the 3 groups of animals described in the legend to Fig. 5. The 20:3 fraction is 8,11,14-20:3. There were 8 animals in each group. Results are shown as the mean ± SEM. (a) $p < .01$; (a′) $p < .05$, group I vs group II; (b) $p < .01$, I vs III; (c) $p < .01$, (c′) $p < .05$, II vs III.

While the proportions of stearic and oleic acids were not significantly changed in the untreated diabetic animal, the insulin treated diabetics showed a significant decrease in stearic acid and an increase in oleic acid. The other change of particular interest was the further drop in arachidonic acid following insulin, rather than the return toward normal seen in the other fatty acids. The increases in palmitoleic and oleic acids with a decrease in stearic acid are consistent with the marked stimulation of Δ9 desaturation in the insulin-treated animals. The significant increase in palmitic acid following insulin is opposite from the expected result from increased Δ9 desaturation and may be the result of insulin-stimulated de novo palmitate synthesis via its stimulatory effect on the acetyl-CoA carboxylase reaction (20). The decrease in linoleic acid following insulin is consistent with the marked stimulation of Δ6 desaturation; however, the expected increase in arachidonic acid did not occur. The further decrease in arachidonic acid following insulin may reflect increased utilization of this important fatty acid not yet compensated for by increased synthesis following repair of the insulin-deficient state.

DISCUSSION

Our studies demonstrated that streptozotocin diabetes in the rat causes changes in liver microsomal lipid fatty acid composition with diminished palmitoleic, oleic and arachidonic acids and increased linoleic and docosahexaenoic acids, consistent with the decreased liver microsomal Δ6 and Δ9 desaturase activities found in these animals. Benjamin and Gellhorn, studying adipose tissue, and Brenner et al., studying adipose tissue and testis, described a significant decrease in palmitoleic acid and and an increase in linoleic acid in alloxan diabetic rats (6,9). Treatment of the animals with insulin for 2 days significantly altered these levels toward normal which suggested that the change in palmitoleic acid was caused by altered synthesis of monounsaturated fatty acids (6). Friedmann et al. (7) found that, although alloxan-diabetic animals had diminished synthesis of [^{14}C] arachidonic acid from injected [^{14}C] linoleic acid, liver phospholipid linoleic and arachidonic acids were unchanged in the diabetic animal. They noted that diabetic animals on a fat-free diet had diminished 5,8,11-eicosatrienoic acid, which suggested that they were unable to normally desaturate oleic acid in the face of essential fatty acid deficiency. Mercuri et al. (8) subsequently showed an increase in linoleic acid and decrease in arachidonic acid in total liver lipids in alloxan-diabetic rats fed fat-free diets. Recently, Worcester et al. (10) reported decreased palmitoleic and oleic acids and increased linoleic acid levels without a change in arachidonic acid in liver and plasma phospholipids of streptozotocin diabetic rats fed a low-fat fructose or glucose diet, although they found only a 15% decrease in Δ9 desaturase in the glucose-fed diabetic animals and no change in the fructose-fed diabetic animals.

Diabetic animals fed ad lib. have polyphagia with significantly greater food intake than control animals, yet gain less weight than control animals. The diminished weight gain of the diabetic animals apparently did not explain the fatty acid composition changes seen, since control animals whose food intake was restricted to achieve a similar weight gain failed to reproduce the changes in fatty acid composition or reduce desaturase activities. The increased food intake per se of the diabetic animals may partially account for some of the alterations in lipid fatty acid composition since limiting food intake in diabetic animals to that of control animals diminished the magnitude of the increase in linoleic acid and the decrease in arachidonic acid while exhibiting the same depression in fatty acid desaturase activities as the diabetic animals fed ad lib. The other changes in fatty acid composition were similar to those in the diabetic animals fed ad lib.

Insulin therapy abruptly reversed and overcorrected the diminished desaturase activities in diabetes. The changes in microsomal fatty acid composition were likewise reversed, with the exception of the decreased arachidonic acid level, which was actually decreased further after insulin. This may be due to an increase in utilization of arachidonic acid in excess of the increase in synthesis. The reversal of the fatty acid composition changes within 2 days following insulin therapy, particularly exemplified by the striking increase in palmitoleic acid from .74 to 6.51% of the total, strongly suggests that these changes in fatty acid composition were indeed caused by the marked increase in desaturase activities.

Moderate restriction of food intake in control animals increased both Δ6 and Δ9 desaturase activities, which may explain the changes in microsomal fatty acid composition seen in these animals. This observation is being further evaluated.

These results indicate that changes in fatty acid desaturation result in alterations in membrane lipid composition. Control of membrane lipid composition probably is multifactorial. In

addition to alterations resulting from changes in Δ6 and Δ9 desaturation, other factors such as effects on Δ5 desaturation, diet, fatty acid chain elongation, membrane lipid degradation and synthesis, and fatty acid oxidation all may be important. The physiological consequences of such changes in membrane composition remain to be determined.

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Activity of Myocardial Lipase Using Natural Edible Oils As Substrates

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ABSTRACT

Rat heart homogenates were tested for their lipolytic activity toward synthetic and natural substrates such as edible oils. Triolein was hydrolyzed very efficiently by myocardial lipase whereas trierucin was not cleaved by the enzyme. Among the natural substrates, safflower oil, which has the highest degree of unsaturation, was hydrolyzed to a greater extent than the other oils. Mustard oil rich in erucic acid formed a poor substrate for the myocardial lipase.

INTRODUCTION

The ingestion of oils from the seeds of Cruciferae family such as rapeseed oil (*Brassica campestris* and *Brassica napus*) and mustard oil (*Brassica juncea*) which contain high amounts of erucic acid has been shown to produce myocardial changes in experimental animals. Prolonged feeding of these oils ultimately leads to myocardial fibrosis (1-4). Short-term feeding produces lipidosis in the heart and skeletal muscle characterized by the accumulation of triglycerides (5-7). The extent of lipidosis depends on the level of erucic acid and also on the age and sex of the experimental animals (8-10).

The mechanism underlying the erucic-acid-induced lipidosis is still obscure, although several possibilities have been suggested. It is well known that the heart accumulates fat when it takes up more fatty acids than it can oxidize. The increased uptake of fatty acids might result from a shift in the plasmatic fatty acid composition in favor of erucic acid and from the weaker binding capacity of albumin for this fatty acid (11,12). In addition, the decreased rate of erucic acid metabolism, compared to palmitic and oleic acids, may be responsible for the accumulation of this fatty acid (13-15). However, all these hypotheses do not fully explain the preferential accumulation of triglycerides containing erucic acid in the myocardium. Hung and Holub (16) suggested that the observed spectacular increase in the triglyceride concentration in the hearts of rats fed rapeseed oil might result from an increased rate of triglyceride synthesis from the excess of available precursors.

Recently, Mersel et al. (17) have observed that intracellular heart lipase does not hydrolyze trierucin and, hence, the accumulation of erucic-acid-rich triglycerides in the heart. These observations were made using synthetic trierucin and it is unclear whether the myocardial lipase behaves similarly toward natural oils which are mixed triglycerides. In this communication, the results of myocardial lipase activities using different natural oils are presented. This report also includes the profile of the fatty acids liberated from various edible oils by myocardial lipase.

MATERIALS AND METHODS

Weanling male CFY rats were sacrificed by cervical dislocation and their hearts excised, weighed and dropped into ice-cold 0.25 M sucrose solution. The hearts were homogenized in a medium containing 0.25 M sucrose and 10 mM Tris/HCl at pH 7.4. Aliquots of this homogenate which contained 10 mg protein/ml served as the source of lipase. The reaction mixture had the following composition: 20 mg of oil or synthetic glycerides, 5 ml of 1.0 M triethanolamine (pH 8.5), 2.5 ml of 20% bovine serum albumin and 2.5 ml of 0.1 M NaCl. It was emulsified prior to enzyme reaction in a hand homogenizer. The lipolytic activity was determined by adding 0.25 ml of homogenate to 0.75 ml of preincubated reaction mixture at 37 C and shaking for 20 min in a Dubenoff shaker maintained at 37 C. The reaction was stopped by adding 8 ml of chloroform/methanol (2:1) mixture. The activity was determined spectrophotometrically by the Duncombe procedure (18). Another set of tubes in which the reaction was stopped by adding 5 ml of Dole's extract was used to determine the composition of the liberated fatty acids. The fatty acids were extracted into 3 ml heptane and the solvent evaporated. Methyl esters of the fatty acids were prepared using methanolic NaOH. The fatty acid profile was determined using a Varian Model 3700 gas chromatograph with a 6 ft column of 15% diethyleneglycol succinate on Chromosorb W, mesh size 80-100; the nitrogen flow rate was 30 ml/min. Column temperature was main-

tained at 185 C and injector and flame ionization detector temperatures were at 250 and 300 C, respectively.

RESULTS AND DISCUSSION

Table I summarizes the results of myocardial lipase activities using synthetic triglycerides, triolein (TO) and trierucin (TE) and also natural oils, e.g., safflower oil (SO), groundnut oil (GNO), rapeseed oil (RSO) and mustard oil (MO) as substrates. The lipolytic activity using trierucin was significantly lower than that seen with triolein ($P < 0.001$). This agrees with the observation made by Mersel et al. (17). With regard to the various oils as substrates, the lipase activity where mustard oil was used was significantly lower than where safflower oil was used ($P < 0.001$). When groundnut oil and rapeseed oil were used as substrates, the activities were similar and were intermediate to that of safflower oil and mustard oil. However, these enzyme activities were not significantly different, either from that using safflower oil or mustard oil as substrates. It was observed from the fatty acid profiles of oils and their lipolyzed products, free fatty acids, (Table II) that fatty acids which had a chain length greater than 20 carbon atoms were released to a lesser extent compared to those with chain length less than 20 carbon atoms. The lipolytic activity apparently was inversely proportional to the amount of fatty acid of chain length more than 20 carbon atoms present in the oil. Beare-Rogers and Nera (19) had observed that the lipid in the myocardiums of young rats fed rapeseed oil and partially hydrogenated herring oil contained high concentrations of docosenoic acid. The deposition of this acid can be explained on the basis of the observation made here—that cardiac lipase hydrolyzes long chain fatty acid containing triglycerides less efficiently. The small difference in the lipolytic activities in this investigation, when groundnut oil and rapeseed oil were used, could occur because rapeseed oil contains a higher proportion of long chain fatty acids than groundnut oil.

The results indicate that the alteration in the lipolytic activity of myocardial lipase could be dependent on the composition of triglycerides with reference to the chain length and to a certain extent on the degree of unsaturation of the constituent fatty acids. In addition, unusual fatty acids such as erucic acid in the triglyceride predominantly affect the rate of lipolysis. Thus, these studies would help in understanding the mechanism of lipidosis seen in animals fed mustard oil.

TABLE I

Myocardial Lipase Activity in Weaning Rats

Substrate	Lipase activity (μmol FFA/mg of protein/hr)
Triolein	0.76 ± 0.052 (19)[a]
Trierucin	0.05 ± 0.027[b] (8)
Safflower oil	0.58 ± 0.055 (7)
Groundnut oil	0.43 ± 0.058 (7)
Rapeseed oil	0.44 ± 0.115 (4)
Mustard oil	0.33 ± 0.034[c] (5)

[a]Number in parentheses indicates the number of observations.

[b]Indicates level of significance between triolein and trierucin, $P < 0.001$.

[c]Indicates level of significance between safflower and mustard oil, $P < 0.001$.

TABLE II

Fatty Acid Composition of Various Oils and Their Products of Lipolysis (Free Fatty Acid)

	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:1
Triolein			100					
After lypolysis	0.5	0.2	99.3					
Trierucin								100
After lypolysis								—
Safflower oil	11.1	0.8	13.1	74.0				
After lypolysis	6.6	Traces	5.3	88.1				
Rapeseed oil	3.9		51.0	28.9	9.7		1.6	4.9
After lypolysis	1.1		68.8	20.6	9.5		—	—
Groundnut oil	12.5	2.7	48.1	33.2		1.9		
After lypolysis	12.9	2.9	48.3	35.8				
Mustard oil	3.0	1.2	14.5	21.3	8.3		6.0	45.7
After lypolysis	3.0	6.5	26.0	30.0	29.1		2.8	2.4

In fatty acids of chain lengths having 18 carbon atoms, there was a tendency for the myocardial lipase to release fatty acids proportional to the degree of unsaturation. However, this was observed only in the safflower and mustard oils. It is possible that the glyceride composition, rather than the unsaturation of fatty acids, determines the release of fatty acids by myocardial lipase. Further investigations are necessary to elucidate this aspect.

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METHODS

A Colorimetric Microdetermination of Peroxide Values Utilizing Aluminum Chloride As the Catalyst

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ABSTRACT

A colorimetric microassay is described for the determination of lipid hydroperoxides. Hydroperoxides are reacted with potassium iodide in the presence of an acid catalyst and liberated iodine is measured. Aluminum chloride, an alcohol-soluble Lewis acid, is used as catalyst. Liberated iodine is measured colorimetrically at 560 nm after addition of starch in 0.01 N hydrochloric acid. The range of the measurement was 0.05-0.5 μmol of hydroperoxides.

INTRODUCTION

Peroxide values (PV) are usually determined by measuring the amount of iodine liberated from potassium iodide through oxidation by peroxides at room temperature in an acetic acid/chloroform medium (1-3). Colorimetric micromethods also have been reported. These include color formation with ammonium ferrous sulfate and ammonium thiocyanide (4), with ferrous chloride and ammonium thiocyanide (5-8), with ferrous chloride and 2,6-dichlorophenol indophenol (9), direct colorimetric determination of iodine (10) or triiodide (11), color formation with diphenylcarbazide (12,13), colored complex formation of titanium and hydroperoxides (14), and colored complex formation of iodine and cadmium ions (15).

In our previous papers (16-18), use of PV test paper was described for a simplified assay of peroxides in lipids. The test paper consisted of a potassium iodide-starch-silica gel sheet. Because silica gel is a Lewis acid, hydroperoxides can react with potassium iodide without an addition of acid as a catalyst. This reaction also was used in a colorimetric microdetermination of PV using a KI-silica gel reagent (19). However, the process was not simple and use of only liquid reagents appeared desirable.

Aluminum chloride, an alcohol-soluble Lewis acid, is used instead of silica gel in this procedure, which uses only reagents that are commonly available. The procedure is reasonably specific for hydroperoxides, is stoichiometric and is adequately sensitive.

MATERIALS AND METHODS

Reagents

Potassium iodide solution was prepared from 2 g of KI in 100 ml of ethanol; aluminum chloride solution was prepared from 2 g of $AlCl_3$ (anhydrous) and 0.02 g of *o*-phenanthroline in 100 ml of ethanol. Starch solution contained 1 g of soluble starch and 20 g of NaCl in 100 ml of water; the mixture was heated until it became clear. The potassium iodate (KIO_3) standard solution was 1 mM.

Procedure

The weighed sample (200 mg or less) or hexane-diluted sample (200 μl or less) was placed in a test tube. Potassium iodide solution (0.5 ml), aluminum chloride solution (0.5 ml) and hexane (1 ml) were added and the mixture was incubated for 5 min at 37 C in a dry block heater. Fifteen ml of 0.01 N HCl and 0.5 ml of starch solution were added and the mixture was shaken vigorously. The solution was transferred into a centrifuge tube, centrifuged for 3 min at 3000 rpm, and then the absorbance of the lower layer was measured at 560 nm. The total vol of the water layer was 16.5 ml. A blank test was run in parallel.

Calibration

Calibration was done by comparing the iodine liberated from potassium iodide through oxidation by potassium iodate standard solution.

Potassium iodate standard solution (0.2 ml),

aluminum chloride solution (0.5 ml) and potassium iodide solution (0.5 ml) were mixed; 0.01 N HCl (15 ml) and starch solution (0.5 ml) were added and the absorbance was read at 560 nm. The total vol was 16.7 ml.

The potassium iodate standard solution (0.2 ml) corresponded to 0.6 μmol I_2, because KIO_3 corresponds to 3 I_2. Therefore, the absorbance corresponding to 1 μmol active oxygen = A/1.2, because I_2 corresponds to 2-O (active oxygen), and PV (meq/kg) = active oxygen (μmol) × 1/sample (g). The A obtained may depend on the kind of starch, but in our experiments, it was 0.95.

This value was corrected for the difference in vol of the sample (16.5 ml) and the standard KIO_3 (16.7 ml). In our experiments, it was calculated to be 0.96.

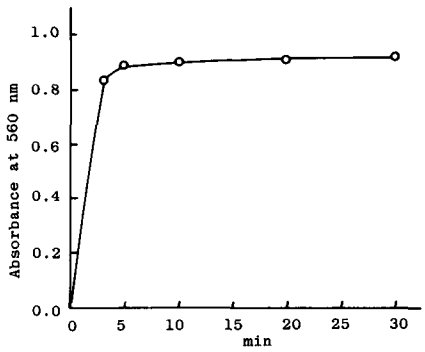

FIG. 1. Time course of iodine liberation with MLHPO at 37 C. A MLHPO hexane solution, 200 μl, was used. A concentration of 2.40 μmol/ml was obtained by titration with thiosulfate (3).

RESULTS AND DISCUSSION

The routine procedures (1-3) for the determination of PV depend on the reaction of potassium iodide with peroxides in acidic solution, followed by titration of the liberated iodine with sodium thiosulfate. Our method makes use of the same principle; however, titration is replaced by colorimetric assay. The use of an alcohol-soluble Lewis acid makes the colorimetric method particularly convenient compared to the KI-silica gel reagent used previously (19). Trifluoroborate also is a good catalyst, but is not commonly available. On the other hand, aluminum chloride is readily obtained and easy to handle. However, aluminum chloride tends to contain trace amounts of iron salts which may interfere in the reaction (11), and therefore, *o*-phenanthroline was added to the solution. Hexane added to the reaction mixture removes the oil after the reaction.

Figure 1 shows the time course of the reaction, i.e., the liberation of iodine, when pure methyl linoleate monohydroperoxides (MLHPO) (20) were used. A 5-min incubation period was sufficient to bring the reaction to completion.

As shown in Figure 2, a linear relationship exists between absorbance and sample size for 25-200 μl of MLHPO in hexane solution. The upper limit of the measurement was 2 μmol active oxygen, that is, PV 10. Larger samples must be diluted with nonoxidized oil or with hexane before PV determination or the sample size should be reduced. The scale of the experiments may be increased or reduced, as long as the ratio of reagents is kept constant.

In the oxidized soybean oil (Fig. 3), the amount of liberated iodine was exactly the

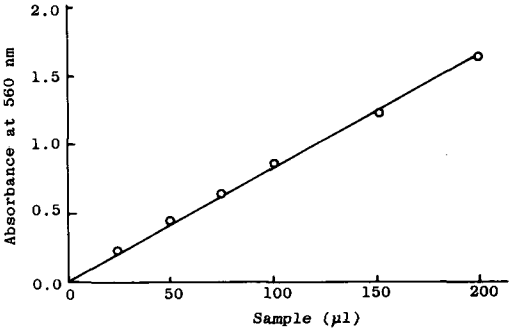

FIG. 2. Relationship between absorbance and sample size. A MLHPO hexane solution, 4.37 μmol/ml obtained from titration with thiosulfate, was used.

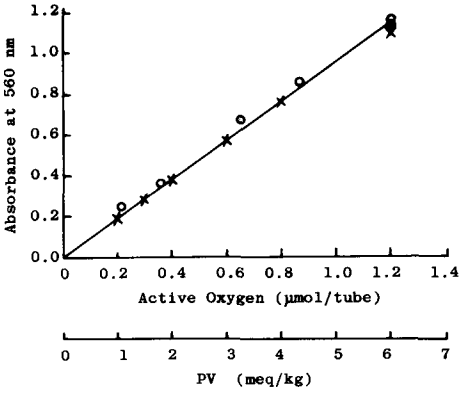

FIG. 3. Relationship between absorbance and different peroxide samples, hydrogen peroxide samples, and potassium iodate standard solution. For each sample, 200 μl was used. • = Potassium iodate standard solution; × = hydrogen peroxide; ○ = soybean oil; PV scale applies only when 200-mg sample is used.

same as that expected from the PV obtained by titration with thiosulfate (3). For calibration of the method, potassium iodate standard solution was used. In this case also (Fig. 3), the amount of liberated iodine in the mixture of potassium iodide, aluminum chloride and potassium iodate in ethanol was exactly the same as that obtained by the routine method, applied to a mixture of potassium iodide, sulfuric acid and potassium iodate in water. Hydrogen peroxide solution standardized with 0.01 N thiosulfate also liberated iodine quantitatively in ethanol. With potassium iodate, no incubation was necessary, but with hydrogen peroxide, the solution had to be incubated for 5 min.

The absorbance of the iodine-starch complex may vary with the source of starch (21), but it can be easily standardized with potassium iodate standard solution. Several kinds of starch were tested and an absorbance range of 0.74-0.95 (see Methods, calibration) was found.

When this method was applied to some arbitrary samples of hexane-diluted MLHPO and oxidized soybean oil, the obtained values (absorbance, $\bar{X} \pm SD$) were 0.916 ± 0.017 and 0.819 ± 0.010 from each of 16 experiments, respectively. Reproducibility was satisfactory.

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Phthalic Ester, an Artifactual Contaminant in Diazomethane Prepared from N-Methyl-N-nitroso-*p*-toluenesulfonamide for the Derivatization of Prostaglandins

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ABSTRACT

During analyses of prostaglandin (PG) methoxime methyl trimethylsilyl (MO-ME-TMS) ether derivatives by gas chromatography, we consistently observed a contaminant peak which we identified as di-2-ethylhexyl phthalate (DEHP). The diazomethane prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide (MNTSA) using the Diazald kit was the major source of DEHP. Several other materials also were significant sources. The concentrations of DEHP in these materials were quantified by gas chromatography-mass spectrometry.

INTRODUCTION

Because of the importance of prostaglandins (PG) in metabolism and in modulating several physiological functions (1), and because their concentrations may be affected by dietary fatty acids (2), standard instrumental methods for the simultaneous identification and quantification of various PG and intermediate endoperoxides in tissues are needed for routine monitoring purposes. Derivatization to form the volatile and stable methoxime methyl trimethylsilyl (MO-ME-TMS) ether derivatives is the preferred approach for detection and quantification of PG (3).

During studies of the tissue levels of PG using gas chromatography, we consistently observed a contaminant peak which interfered with the identification and measurement of some PG peaks. Using gas chromatography-mass spectrometry (GC-MS), we have identified this contaminant as di-2-ethylhexyl phthalate (DEHP) and the diazomethane, prepared from N-methyl-N-nitroso-*p*-toluene-sulfonamide (MNTSA) using the Diazald kit, is the major source of this compound.

METHODS

Samples of commercial N-methyl-N-nitroso-*p*-toluene-sulfonamide (MNTSA) were obtained from the Aldrich Chemical Co. (Milwaukee, WI) and the Sigma Chemical Co. (St. Louis, MO), and N-methyl-N-nitroso-N-nitroguanidine (MNNG) from Aldrich Chemical Co. These reagents were used without further purification. The ethereal diazomethane was prepared from the MNTSA according to the procedure of Aldrich Diazald Technical Information (product no. D-2800-0) (4) using a Diazald Kit, and from the MNNG by the Fales et al. procedure (5). These diazomethane solutions were stored in screw-capped flasks at −40 C and freshly prepared every 2 weeks.

The oximation reagent O-methylhydroxylamine hydrochloride and silylation reagent bis-(trimethylsilyl) acetamide (BSA) were purchased from Applied Science (State College, PA). The 5α-cholestane, used as an internal standard, was supplied by Sigma Chemical Co. (St. Louis, MO). The prostaglandins (e.g., E_2, A_1, A_2) were obtained from Dr. John Pike, The Upjohn Co. (Kalamazoo, MI). All the solvents used were analytical grade from Mallinckrodt (St. Louis, MO). The authentic fatty acid methyl esters were purchased from Nu-Chek-Prep. (Elysian, MN). Authentic di-2-ethylhexyl phthalate (DEPH) was obtained from Pfaltz and Bauer, Inc. (Stanford, CT).

Aliquots of PG (50-100 μg) in ethanol were transferred to reaction vials. Following evaporation of the ethanol, 100-200 μl ethereal diazomethane was added to these vials. The reaction was allowed to proceed for 5 min and the solvent was then evaporated. The PG methyl esters were then oximated and silylated according to Fitzpatrick's procedures (3).

GC was performed with a Hewlett-Packard (HP) 5830A instrument equipped with a flame ionization detector modified for capillary column and a HP-18850A GC terminal. Two different capillary columns were used, a stainless steel capillary column (150′ x 0.01″) (Perkin-Elmer, CT) was wall-coated with OV-101 and the glass capillary column (40 m x 0.30 mm) was coated with the same liquid phase.

A splitless mode was used for sample injec-

tion. The purge time was adjusted for 0.3 min after injection. The injection port and detection port temperatures were 250 and 300 C, respectively. The initial column temperature was 45 or 60 C, the temperature was held for 5 min and then programmed at 25 C/min for 5 min and followed by 10 C/min until 245 or 260 C was reached. The flow rates of the carrier gas (N_2) was 1.2 ml/min and the make-up gas (N_2) 30-40 ml/min. The flow rates of the air and hydrogen were 200 ml/min and 20 ml/min, respectively.

The GC-MS was performed on a Finnigan GC-MS 3300 unit and a Data System 150 from Systems Industries (Sunnyvale, CA). A 12' x 1/8", 3% OV-101 glass column was programmed from 200 to 295 C at 20 C/min. Chemical ionization was used to determine the molecular weight (MW) of the unknown. The chemical ionization methane reagent gas pressure was set at 400 microns.

For accurate identification and quantification of the unknown, several analyses were performed using a Hewlett-Packard 5992B GLC-MS system using the technique of selected ion mass spectrometry. A 2' x 1/4" glass column packed with 3% SE-30 (80-100 mesh) was used for the separation of the unknown compound by GC. The column was run isothermally at 240 C using helium carrier gas at 15 ml/min.

RESULTS AND DISCUSSION

Both the glass and stainless steel capillary columns efficiently separated the PG derivatives. However, an unknown peak consistently appeared on chromatograms of reagent blanks (Fig. 1A) as well as those of authentic PG samples (Fig. 1B). Overlapping of peaks or only partial separation from the unknown peak were obtained when PGA derivatives were analyzed on the stainless steel capillary column (Fig. 1C).

After appropriate systematic analyses of reagents, solvents, reaction vials, containers and the Diazald kit, we found that the ethereal diazomethane, the diethyl ether, the 95% ethanol, the cap of the diazomethane storage flask, the cap of the diethyl ether tank, the cap of the 5 gal 95% ethanol tank, the vials for the prostaglandin samples, the rubber thermometer holder and the Tygon tubing R-3603 of the Diazald kit and the glassware components of the Diazald kit all contained the contaminant. The Tygon R-3603 tubing and the ethereal diazomethane contained the highest concentration of the unknown peak. Most of the contaminant in the ether originated from the diazomethane prepared from the MNTSA using the Diazald kit. Further analyses were conducted to confirm the identity of the unknown.

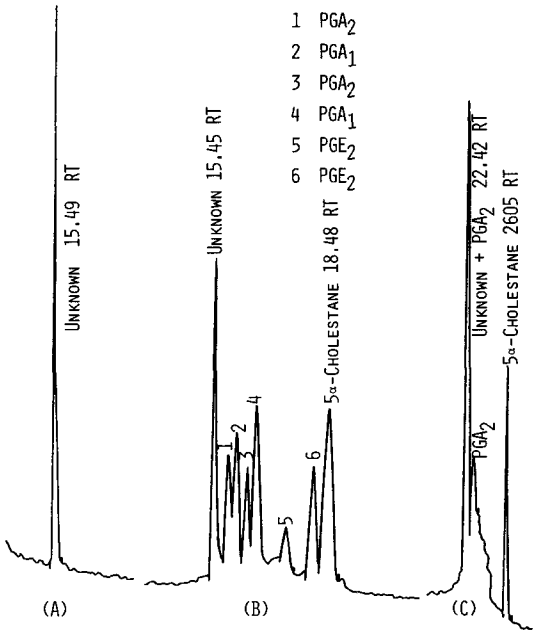

FIG. 1. Gas chromatograms of (A) reagent blank run by 40 m x 0.30 mm OV-101 glass capillary column indicating the presence of the unknown (B) prostaglandins A_2, A_1 and E_2 MO-ME-TMS anti- and syn-isomers coeluted with the unknown on 40 m x 0.30 mm glass capillary column; 5α-cholestane was used as an internal standard (C) overlapping of one of the PG A_2, Mo-ME-TMS isomer with the unknown run by a 150' x 0.01" OV-101 stainless steel capillary column. See the Methods for the details of running condition.

Extraction of the MNTSA, obtained from Aldrich and Sigma, with hexane and GC analyses showed that these extracts contained 3 and 2 peaks, respectively, none of which corresponded to the unknown peak in the distilled ethereal diazomethane. Analyses of these peaks by GC-MS showed them to be *p*-toluene sulfonyl azide, N-methyl-*p*-toluene-sulfonamide and ethyl-*m*-methylphenylsulfonyl acetate, respectively. These data indicated that the MNTSA did not contain the unknown compound.

When the ethereal diazomethane was prepared from N-methyl-N-nitroso-N-nitroguanidine (Aldrich Chemical Co.), the unknown peak was not observed.

From these experiments, it was apparent that the diazomethane prepared from MNTSA was the source of the unknown. When increasing amounts of diazomethane prepared from MNTSA was injected into the GC, a linear recovery of the unknown was obtained.

Ethereal diazomethane prepared from MNTSA using the Diazald Kit was dried under

nitrogen. The yellowish, sticky, oily residue was weighed and dissolved in a known amount of hexane.

The mass spectra data (Fig. 2) indicated that the MW of the unknown was 390, corresponding to a formula of $C_{24}H_{38}O_4^+$ (abundance at m/e 391 was the parent ion after protonation by CH_5^+). The most abundant peak at m/e 149 [M-141] corresponded to the rearrangement ion from phthalate anhydride formed by loss of water from the diacid fragment at m/e 167 [M-223] (6). The fragment at m/e 279 [M-111] was derived by the loss of a 2-ethylhexyl group from the parent molecular ion. This was further supported by the fragment of m/e 112 [M-278] corresponding to a 2-ethylhexene hydrocarbon from the alcohol moiety.

From the information available, the mass spectrum indicated a structure of a di-2-ethylhexyl phthalate (DEHP). The amount of DEHP in the reagents, solvents and apparatus was determined by GC-MS using the selected ion monitoring technique and data are summarized (Table 1).

Phthalate esters, which are widely used as plasticizers, are potentially toxic (7), and thus are of concern to public health and environmental personnel. They are metabolized and converted to water-soluble products and excreted by rats (8). The occurrence of these esters in commercial solvents and laboratory equipment has been reported (9). DEHP is widely used as a plasticizer (10) to impart desired flexibility and softness to plastic products.

MNTSA has been used as a precursor for diazomethane for a long time. The presence of DEHP in the diazomethane has not yet been reported, probably because high temperatures (> 230 C) and a relatively nonpolar liquid phase are necessary for its elution from gas chromatographic columns. In our experience with several liquid phases (e.g., Silar 10C, EGSS-X, FFAP, OV-17 and OV-101), the OV-101 gave the best resolution and recovery of DEHP. The other 3 phases were too polar and DEHP was not eluted as a symmetric peak. The failure to observe DEHP contaminants during analysis of fatty acid methyl esters prepared using diazomethane from MNTSA is perhaps because the column temperature is too low to volatilize the compound and/or the liquid phase used is too polar for elution. In our work with prostaglandin derivatives, high temperature (> 230 C) and nonpolar liquid phases are required and these cause the elution of DEHP. The sensitivity required for the accurate quantification of PG makes it extremely important to eliminate contamination or artifacts such as that demonstrated by our present observation.

In order to avoid contamination by DEHP in the diazomethane prepared by the Diazald Kit, all the materials which are made of plastic or solvents which have had contact with plastic containers should be fastidiously avoided. Glassware used to prepare or store diazo-

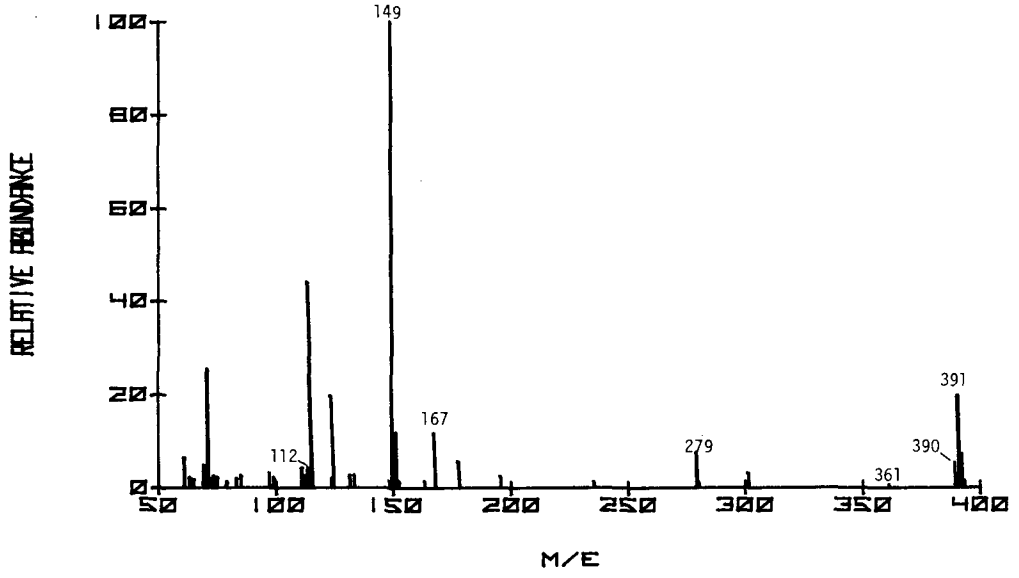

FIG. 2. Chemical ionization mass spectrum of di-2-ethylhexyl phthalate (DEHP) isolated from ethereal diazomethane.

TABLE I

Concentrations of Di-2-ethylhexyl Phthalate (DEHP) from Different Sources of Contamination As Quantified by GC-MS Selected Ion Monitoring Technique

Sources of DEHP	Concentration (μg/ml)
Tygon tubing R3603[a]	1.5×10^{2}
Ethereal diazomethane prepared by Diazald Kit	4.3×10^{-1}
Redistilled ethyl ether (100 ml) washing residue of all the glassware from a new set of Diazald Kit	6.5×10^{-2}
Cap of the diazomethane storage flask[a]	5.1×10^{-2}
Cap of the 5 gal 95% ethanol tank	4.2×10^{-2}
Thermometer holder of the Diazald Kit[a]	3.8×10^{-2}
Cap of the diethyl ether tank[a]	2.6×10^{-2}
Diethyl ether	6.2×10^{-4}
Vials for prostaglandin samples[a]	4.3×10^{-4}
95% Ethanol	9.0×10^{-5}

[a]Extracted by 20 ml redistilled diethyl ether.

methane should be thoroughly cleaned with redistilled ether followed by DEHP-free *n*-hexane. It also is recommended that the plastic tubing supplied with the Diazald Kit should be replaced with a plasticizer-free tubing or the ethereal diazomethane collected through this tubing should be discarded, rather than combined with the other portion collected as is currently suggested by the manufacturer (Aldrich, Diazald Technical Information).

ACKNOWLEDGMENT

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COMMUNICATIONS

The Identity of the Cholesteryl Esters in Human Milk[1]

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ABSTRACT

Milk samples were collected from 11 mothers who were at least 4 weeks postpartum. The amounts of fat and the fatty acid compositions of cholesteryl esters (CE) and triacylglycerols (TG) in the milk were determined. The mean concentration of total milk lipid was 3.01 gm/100 ml of milk ±.42 SD. The major fatty acids esterified with CE and TG were 16:0, *cis* 18:1 and 18:2. The patterns were similar except for a greater proportion of *cis* 18:1 in the CE. The major *trans* fatty acid detected was the 18:1 isomer which accounted for 4.48% of the TG fatty acids and 2.96% of the CE fatty acids.

INTRODUCTION

Among many groups in the United States and elsewhere, there is a reemergence in the popularity of breast feeding. With the American Academy of Pediatrics (1) publicly endorsing the use of human milk as the sole source of nutrients for an infant during the first 6 months of life, the trend for increased breast feeding should continue. As the use of human milk increases, we should know, to the extent possible, the nutrient content of this milk. It is therefore important that we have a detailed knowledge of the composition of human milk and an understanding of the factors which may affect it.

Unfortunately, our knowledge of the composition of human milk, other than concentrations of major classes of nutrients, is very limited. In our recent review on the lipids in human milk, we noted that there was little or no information available on several lipid classes (2). One of these neglected classes was the cholesteryl esters (CE) of human milk. Bracco (3) reported that sterol esters accounted for ca. 15% of the total sterol in human milk and contained a high proportion, amounts unspecified, of 18:2 and 18:3. However, there are no quantitative data available on the fatty acids associated with CE in human milk or identity of other fatty acids. The amounts of total cholesterol in human milk have been reported as ranging from 200 to 564 mg/100 g of lipid (4).

Our objective was to identify the fatty acids of CE and compare them to those in triglycerides (TG). In previous work with milk from other species, differences were found in the fatty acid compositions of CE and TG (5).

MATERIALS AND METHODS

Eleven healthy mothers 4-48 weeks postpartum donated milk. General health questionnaire and dietary recall forms were used to eliminate volunteers with factors known to affect lactation. Milk samples were collected between 9:30-12:00 a.m. at least 1 hr after the infants' last feeding. One breast was emptied with the aid of an electric breast pump (Egnell, Inc., Gary, IL). The milk was immediately placed on Dry Ice and then transported in a styrofoam container to the laboratory for storage at −70 C.

On the day of analysis, milk quickly was thawed to 25 C and a 10-ml aliquot taken. The lipids were extracted with 90 ml chloroform/methanol (2:1). After removing the chloroform layer, the supernatant was washed with an additional 60 ml of chloroform. The chloroform layer and wash were combined and evaporated under reduced pressure. Total lipids were determined gravimetrically.

Polar and nonpolar lipids were separated on a column containing Unisil (Clarkson Chemical Co., Williamsport, PA) as recommended by Rouser et al. (6). CE and TG were isolated on Silica Gel G thin layer chromatographic plates developed with petroleum ether/ethyl ether/glacial acetic acid (90:30:2). When necessary, further purification of CE and TG was achieved by a second plating on Silica Gel G plates developed with petroleum ether/ethyl ether (95:5). CE and TG were eluted from the absorbent with chloroform/methanol (9:1). The infrared spectrum of CE isolated from human

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milk resembled the spectrum of pure CE.

The fatty acids associated with the CE and TG were transesterified with sodium methoxide (7). The methyl esters of the fatty acids were analyzed using gas liquid chromatography (GLC). The column was packed with 15% OV-275 on 100/120 Chromosorb P AW-DMCS (Supelco, Inc. Bellefonte, PA). Identifications of fatty acids were based on retention times of known fatty acids and carbon number plots. The fatty acids associated with CE and TG are reported as weight percentages. The presence of *trans* fatty acid in CE and TG was verified by absorbance of their methyl esters at 10.3 μ on the infrared spectrum (8). The composition of the fatty acids associated with CE and TG were compared statistically using multivariant analysis (9). Individual fatty acids were compared using a t-test (10).

RESULTS AND DISCUSSION

The mean vol of milk collected was 75.3 ml ± 53.6 SD. Average total lipid in the 11 samples was 3.01 gm/100 ml of milk ± .42 SD.

The composition of the fatty acids esterified as TG and CE is given in Table I. The major fatty acids identified with each class of lipids were similar. However, the proportion of individual fatty acids differed significantly ($P < .05$) between lipid classes. The major difference was due to *cis* 18:1. The proportion of *cis* 18:1 in CE averaged 41.12%, which was significantly ($P < .05$) greater than the 34.89% observed in TG. Keenan and Patton (5) reported a similar increased 18:1 associated with CE in goat and cow milk.

Keenan and Patton also observed a greater proportion of odd-chain fatty acids esterified to cholesterol than to TG in the milk from goats and cows. We did not consistently find measurable amounts of odd-chain fatty acids. In some TG samples, trace amounts (<.1%) of 15:0 were detected. Odd-chain fatty acids probably were associated with CE but, because of the small concentration of CE in our samples, were not detected.

During the past 20 years, the consumption of vegetable oils and partially hydrogenated vegetable oils has increased in America (11). The hydrogenation of vegetable oils results in the formation of several *trans* isomers of the fatty acids. It has been estimated that Americans who have increased their intake of polyunsaturated fatty acids could be consuming up to 7% of total dietary calories as *trans* fatty acids (11). The *trans* fatty acids find their way into human milk, and amounts of 2-18% of the total fatty acids have been reported (4). In this study, *trans* isomers of 16:1 and 18:1 were tentatively identified (Table I). The quantities of *trans* 18:1 in TG averaged 4.48%; range 2.47-7.45%. The *trans* 18:1 in CE averaged 2.96%; range 0-6.76%. The percentage of *trans* 18:1 in CE did not increase with the proportion of *cis* 18:1 in CE, suggesting a preferential esterification of the *cis* isomer to cholesterol.

In conclusion, the major fatty acids esterified to CE in human milk were identified and found to be similar to those in TG. There was a greater proportion of *cis* 18:1 in CE than in TG. The major *trans* fatty acids were 16:1 and 18:1 isomers. The smaller proportion of *trans* fatty acids associated with milk CE suggests a

TABLE I

Fatty Acids Associated with Triacylgylcerols and Cholesteryl Esters in Human Milk

Fatty acid	Triacylglycerols		Cholesteryl esters	
	Mean	SD	Mean	SD
	Wt %			
8:0	0.17	0.07	Tr[a]	
10:0	1.34	0.48	0.59	0.74
12:0	5.16	1.52	3.19	2.29
14:0	5.41	1.22	4.75	1.91
16:0	21.52	2.19	23.83	5.26
16:1 *trans*	0.39	0.27	ND[b]	
16:1 *cis*	3.37	0.69	1.48	1.40
18:0	7.99	1.55	8.03	3.43
18:1 *trans*	4.48	1.33	2.97	2.60
18:1 *cis*	34.89	2.31	42.71	11.66
18:2	14.18	4.43	12.38	7.35
18:3	0.49	0.55	Tr	
20: polyene and greater	.90	0.83	Tr	

[a]Less than 0.1%.
[b]None detected.

decreased incorporation. The competition of *trans* fatty acids with other fatty acids for esterification with cholesterol should be investigated further.

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The Effects of Dietary 9-*trans*,12-*trans*-Octadecadienoate on Composition and Fatty Acids of Rat Lungs

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ABSTRACT

Dietary *trans,trans*-linoleate (*trans* 18:2), when fed to rats in increasing amounts, caused a reduction in lung weights, particularly at very high dietary concentrations. The fatty acids of the phospholipids and triglycerides were altered. The percentage of oleic and arachidonic acid decreased as dietary *trans* 18:2 was increased. Eicosatrienoic acid (20:3) appeared in the phospholipids of lungs from rats receiving 100% dietary *trans* 18:2, but its concentration was much lower than in lungs from rats on an essential fatty acid deficient diet, indicating that *trans* 18:2 inhibited the enzymes synthesizing 20:3.

The biosynthesis and secretion of the lipid constituents of the pulmonary surfactant system, which is required to facilitate gas exchange, is critical for lung function (1,2). In addition, the production of prostaglandins, endoperoxides and prostacyclin and their precursors is extremely important for hemodynamics and pulmonary performance (3-5). There is limited information concerning the effects of dietary fatty acids on lung lipids and particularly the effects of *trans* acids on the levels of linoleic and arachidonic in pulmonary tissue. Processed fats and oils, which contain varying amounts of *trans* isomers of fatty acids (6), are a significant source of dietary lipids and they affect the fatty acid composition of vital organs (7-10). Herein we report the effects of high levels of *trans,trans*-linoleic acid on lung lipids, particularly the levels of linoleic and arachidonic acid in the lipid classes.

METHODS AND MATERIALS

Animals and Diet

Weanling male Sprague-Dawley rats weighing 40-50 g were fed a basal fat-free diet (11) supplemented 5% by weight with one of 4 fats. Highly purified methyl esters of 9-*cis*,12-*cis*-octadecadienoate, 9-*trans*,12-*trans* octadecadienoate (Nu-Chek-Prep, Elysian, MN) and hydrogenated coconut oil (Nutritional Biochemicals, Cleveland, OH) were used as dietary sources of fat. The rats were divided into 4 groups of 12 each and fed on a diet containing *cis,cis*-linoleate (*c*,c-18:2, treatment A), a mixture of equal amounts of *trans,trans*-linoleate and *cis,cis*-linoleate (treatment B), *trans,trans*-linoleate (*t*,t-18:2, treatment C) or hydrogenated coconut oil (treatment D) as sole sources of dietary fats, respectively. All diets except the HCO diet were mixed every 3-4 days and stored in a cold room (5 C). Fresh diet was provided daily to each group. After 12 weeks, rats from each group were lightly anesthetized with ether. After complete bleeding, the lungs were removed from the rats, washed, weighed, frozen in liquid nitrogen and stored at −20 C until analyzed.

Lipid Analyses

The lipids were extracted from homogenized lung tissue with chloroform/methanol (2:1, v/v) according to the Folch et al. method (12). The lipids dried under nitrogen were weighed and dissolved in chloroform (containing 0.01% BHT) to give a known concentration of lipids. Cholesteryl esters, triglycerides, free fatty acids and phospholipids were separated by thin layer chromatography (TLC) on activated Silica Gel G plates with standard references on both edges (13). The plates were developed in petroleum ether/ethyl ether/acetic acid (70:30:1), dried and sprayed with 0.2% of 2′,7′-dichlorofluorescein. The lipid classes were scraped from the TLC plates, extracted (triglycerides and cholesteryl esters were eluted with diethyl ether containing 1% acetic acid; free fatty acids and phospholipids were eluted with chloroform/methanol [2:1]), filtered through Whatman No. 1 paper, and concentrated under nitrogen. The phospholipids were quantified colorimetrically (14). Fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) (Hewlett Packard Model 5830A) using a silanized stainless steel column (1.9 m x 2 mm) with 10% EGSS-X coated on gas-Chrom Q 100-120 mesh (Applied Science, Inc.). The temperatures of the injection port and the detector were 250 and 300 C, respectively. The temperature of the column was programmed from 170 to 210 C, at a rate of 1.5 C/min. A glass capillary column (30 m long, Perkin Elmer) wall-coated with Silar 10C was used for the separation of the *cis,cis*- and *trans,trans*-isomers of linoleic acid. The flow rate of carrier gas nitrogen was 1 ml/min for the glass capillary column, and 15

ml/min for the packed column. The identities of GLC components were determined as described earlier (15). Protein was quantified by the Lowry et al. method (16) using bovine serum albumin as a standard.

RESULTS

The rats receiving hydrogenated coconut oil showed the typical symptoms of essential fatty acid deficiency (7,9,11,17). This was exacerbated in the rats on *trans,trans*-linoleate as reflected in the body weights (Table I). The lung weights decreased with the intake of *trans*-linoleate, particularly on diet C. The protein and lipid content of the pulmonary tissue in rats on the different dietary fatty acids revealed no major changes. The phospholipids were lowest in lungs from rats receiving dietary *cis,cis*-linoleate.

The fatty acid composition (percentage distribution) of the total lipids and triglycerides in lungs from rats fed on diets D and C (Table II) showed patterns typical of EFA deficiency, i.e., elevated levels of palmitoleic acid, oleic acid and eicosatrienoate (20:3[*n*-9]) and lower levels of linoleate (18:2[n-6]) and arachidonic acid (20:4). Higher levels of dodecanoic and tetradecanoic acids occurred in the lipids of lungs from rats fed diet D, reflecting the presence of these fatty acids in the coconut fat. Lipids of the lungs from rats on diet C accumulated significant levels of linolelaidate (18:2-*t,t*). The concentrations of linoleate (18:2[n-6]) and arachidonate were elevated in the total lipids from rat lungs of diet A. The fatty acid composition of lung lipids of rats fed diet B exhibited a pattern intermediate between those of lungs from rats on diets A and C.

The fatty acid composition of the phospholipids differed from that of the triglycerides most noticeably with respect to palmitic, oleic and *trans,trans*-linoleate levels. The phospholipids (PL) composed mostly of phosphatidylcholine (PC) (55%) had a high content of palmitic acid (16:0) in all groups, reflecting the fact that dipalmitoyl lecithin is the major species of PL (1). The relative percentage of 16:0 was somewhat depressed in PL from rats on diets B, C and D. In the EFA-deficient rats, groups C and D, there was a marked increase in palmitoleic and oleic acid. Eicosatrienoic acid (20:3[n-9]) accumulated in PL of group D and to a much lesser degree in rats from group C, suggesting that the dietary *trans*-18:2 may have impaired the elongation and further desaturation of 18:2 (9). The *trans,trans*-linoleate was excluded from the phospholipids to a greater extent than in the case of the triglycerides, particularly in rats on diet B.

The palmitic acid was markedly depressed in the cholesteryl esters (CE) of rats on diets lacking dietary linoleate. There was a marked increase in palmitoleic and oleic acids, particularly in the EFA-deficient animals. A 2-fold higher level of *cis,cis*-18:2 in group B compared to group A suggested that, in the presence of *trans,trans*-18:2, there was some increase in selective incorporation of *cis,cis*-18:2 into CE. There apparently was a tendency to exclude *trans,trans*-18:2 from CE as indicated by the low level of this fatty acid in lungs from rats on diet B. In the absence of dietary *cis,cis*-18:2, i.e., diet C, *trans,trans*-18:2 accumulated in CE. As observed with the PL, the level of arachidonic acid was higher in the CE of lungs from rats on dietary hydrogenated coconut oil.

The actual concentration of individual fatty acids (mg/g lung tissue) in the lungs was determined to facilitate quantitative comparisons (Table III). In general, the trends noted for the percentage distribution reflected the changes in actual concentrations. There was a marked increase in pentadecanoic acid in lungs from EFA-deficient animals. The monoenoic acids were increased, except in the case of C where

TABLE I

The Body, Lung Weights and Composition of Rats after 12 Weeks on Different Dietary Fats

	Dietary fat			
	Linoleate (A)	50% Linolelaidate (B)	100% Linolelaidate (C)	Hydrogenated coconut oil (D)
Body weight (g)	373 ± 26	334 ± 26	228 ± 26	308 ± 29
Lung weight (g)	1.95 ± 0.16	1.63 ± 0.20	1.49 ± 0.03	1.87 ± 0.20
Protein mg/g	128 ± 11	135 ± 7.0	125 ± 6.0	127 ± 7.0
Total lipid mg/g	96.8 ± 4.9	117.8 ± 5.6	96.26 ± 7.5	109.9 ± 5.6
Phospholipids mg/g	44.0 ± 2.6	54.6 ± 4.0	47.17 ± 5.2	57.8 ± 6.0

TABLE II

Fatty Acid Composition of Pulmonary Lipids Isolated from Rats on Different Dietary Fats

Fatty acids (wt %)	Total lipids				Triglycerides				Phospholipids				Cholesterol esters			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
12:0	0.13	0.14	----	3.82	0.20	2.07	0.24	2.45	0.27	2.60	----	0.13	1.22	0.42	----	0.51
14:0	2.58	2.41	3.62	5.56	2.74	2.27	2.84	5.02	3.49	3.24	3.79	4.35	3.12	2.26	2.54	3.2
14:1	0.34	0.22	----	----	----	----	----	----	----	----	0.16	0.41	1.22	1.02	0.80	3.0
15:0	----	0.15	6.41	4.40	----	----	7.62	3.98	----	----	0.79	2.85	----	----	0.50	2.5
16:0	31.88	30.66	33.62	29.14	31.30	27.60	22.88	28.50	62.40	56.78	47.29	45.89	50.00	33.66	29.22	26.0
16:1	8.19	8.82	11.52	13.48	7.71	7.63	10.25	10.69	6.18	5.20	14.36	11.91	3.20	3.89	18.30	10.10
17:0	----	----	1.69	0.33	----	----	0.46	----	----	----	2.80	0.44	1.01	----	0.25	0.74
18:0	6.57	5.97	5.94	3.43	5.58	4.46	4.66	4.84	7.31	6.99	3.56	6.24	14.42	5.15	4.69	6.5
18:1c	24.23	29.37	21.33	27.75	30.89	36.03	39.49	40.70	10.97	14.83	17.63	15.33	13.04	20.70	26.90	31.20
18:2*t,t*	----	5.78	5.41	----	----	5.28	6.68	----	----	0.98	3.43	----	----	1.3	6.32	----
18:2*c,c*	19.11	11.22	2.09	2.73	20.18	11.63	1.25	0.86	3.53	6.01	1.71	0.94	8.25	18.65	3.79	4.10
18:3(n-6)	----	----	0.39	0.13	0.11	----	0.86	0.45	----	----	0.42	0.52	0.34	2.82	1.60	1.4
20:2(n-6)	0.20	0.20	0.48	0.08	0.23	----	0.40	0.30	----	----	0.17	0.11	0.66	0.25	----	0.54
20:3(n-6)	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	0.69
20:3(n-9)	----	----	1.40	2.78	----	----	----	0.15	----	----	1.71	4.50	----	----	1.	0.80
20:4(n-6)	5.50	4.46	0.96	2.09	0.17	0.81	0.15	0.30	1.79	1.14	1.38	2.64	1.16	0.97	0.84	3.2
20:5(n-3)	----	----	0.36	0.62	----	0.33	----	0.13	----	----	0.27	0.78	-----	1.56	----	0.58
22:4(n-6)	1.00	0.59	2.52	1.00	0.17	----	0.35	0.22	1.14	0.84	----	0.36	1.08	1.11	----	0.4
22:5(n-3)	----	----	0.52	0.61	----	0.76	----	0.51	0.44	0.40	0.19	1.18	----	3.65	1.30	4.0
22:5(n-6)	0.29	----	----	0.08	----	----	----	----	----	----	----	0.13	----	----	----	----
22:6(n-3)	----	----	1.43	1.99	0.67	1.11	1.84	0.88	1.19	1.00	0.35	0.37	2.40	2.16	0.34	0.9

TABLE III

Concentration of Major Fatty Acids Associated with Total Lipids and Phospholipids in Lung Tissue from Rats on Different Dietary Fats[a]

Fatty acid	Concentration (mg total fatty acid acid/g lung tissue)				Concentration (mg phospholipid fatty acid/g lung tissue)			
	A	B	C	D	A	B	C	D
12:0	----	----	----	3.6	----	----	----	----
14:0	2.2	2.4	2.2	5.3	1.1	1.2	1.3	1.8
15:0	----	0.1	5.4	4.1	----	0.2	0.2	1.2
16:0	27.0	31.4	28.2	27.6	21.0	23.2	17.0	20.0
16:1	6.9	9.0	9.4	12.8	2.1	2.0	5.1	5.2
18:0	6.2	6.1	4.1	3.2	2.5	2.9	1.3	2.7
18:1	20.4	30.1	17.0	26.3	3.7	6.1	6.3	6.6
18:2*t,t*	----	5.9	4.3	---	----	0.5	1.4	----
18:2*c,c*	16.1	11.5	1.7	2.6	1.2	2.4	0.5	0.7
20:3(n-9)	----	----	1.2	2.6	----	----	0.6	2.0
20:4(n-6)	4.6	4.6	0.8	2.0	1.3	0.5	0.5	1.2
20:5(n-3)	0.8	----	0.2	0.6	----	----	0.5	t
22:4(n-6)	0.8	0.7	2.1	0.9	0.4	t	t	t
22:6(n-3)	----	----	4.3	1.9	0.1	0.4	t	t

[a]A, B, C, D as described in Methods.

oleic acid was depressed. The concentration of the essential fatty acids 18:2 and 20:4 were significantly reduced in the rats on EFA-deficient diets.

In the case of fatty acids associated with the PL, palmitic acid, the major component, was not affected very much by dietary fatty acids, except in lungs from rats on the *trans,trans* diet which possessed lower concentrations of palmitic acid. The palmitoleic acid level was enhanced in C and D and oleic acid in B, C and D. Compared to the levels in total lipids, the concentration of *trans,trans*-18:2 was much lower in PL from lungs of rats on dietary treatments B and C. The PL from lungs of rats on diet B contained twice the level of *cis,cis* 18:2 compared to those on diet A.

DISCUSSION

The effects of *trans*-linoleate and EFA-deficient diets on growth and organ weights, heart and kidney have been reported (10,18). The *trans,trans*-linoleate resulted in lower body and lung weights. Unlike the data for heart and kidney, where distinct trends were observed, the data on protein and lipid content of lungs showed no clear patterns of change in response to dietary fat.

The changes in fatty acids are in agreement with earlier observations (7-10), indicating increased synthesis of monoenoic acids and eicosatrienoic acid in EFA-deficient animals. However, the increase in levels of these were less in rats on diet C compared to D. This is in accord with the knowledge that *trans,trans*-linoleate inhibits acyl desaturases (9). In the PL and CE, it appeared that in the presence of *cis,cis*-linoleate the *trans,trans*-linoleate was excluded. This is consistent with the reported discrimination against incorporation of *trans,trans* into cholesteryl esters of egg yolk (19) and cardiac tissue (8).

Despite the variations in other components, the consistency of the palmitic acid concentration in lungs from rats on different diets probably reflects the knowledge that dipalmitoyl lecithin is the critical functional lipid of lung tissue (1) and thus is conserved. The low concentration of arachidonate associated with lung PL, especially in rats receiving *trans,trans*-linoleate, might result in diminished prostaglandin production as observed for platelets, because the concentration of precursor fatty acid apparently affects the amount of prostaglandin synthesized (11). Current research is concerned with determining the effects of *trans* fatty acids on prostaglandin synthesis in lung tissue.

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Fatty Acid Profile of Myocardial Lipid in Populations Consuming Different Dietary Fats

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ABSTRACT

Human hearts from medico-legal autopsies were obtained from 3 regions of India where the edible oils used are different. These 3 population groups consumed either mustard oil (Calcutta), coconut oil (Trivandrum) or peanut oil (Madras). Among the saturated fatty acids, lauric acid was found only in the myocardium of subjects from Trivandrum (3.5% of the total fatty acids [TFA]), heptadecanoic acid only in the samples from Madras (1.4% TFA) and arachidic acid only in those of Calcutta (1.9% TFA). Erucic acid was present only in the samples received from Calcutta and acted as a marker of mustard oil consumption. It had a mean concentration of 5.6% TFA. From the level of erucic acid present in the myocardial lipid, it is apparent that, at the level of intake of mustard oil prevalent in India, the risk of developing myocardial fibrosis is slight.

INTRODUCTION

Myocardial fibrosis as a result of prolonged feeding of rapeseed oil (*Brassica campestris*) has been shown in various experimental animals (1-3). The high content of erucic acid in this oil has been determined to be a causative factor (4). Mustard oil (*Brassica juncea*) (which also contains similar amounts of erucic acid), when fed to monkeys at a level of 20% in the diet for a period of ca. 60 weeks, produced myocardial fibrosis in a considerable number of animals (5). Although the level of oil used in this study was very high and habitual Indian diets do not contain such a high level (6), we considered it important to explore the relevance of these observations made in the subhuman primates to the human situation. We decided that a comparative study on the morbid anatomy of hearts obtained from autopsies from subjects living in a region where mustard oil was the habitual edible oil and from subjects whose diets never included mustard oil would provide useful information. Histopathological examination of the hearts showed essentially similar findings in these 2 types of populations (7). In this communication, we present data on the fatty acid profile of lipids extracted from the myocardiums of subjects whose habitual diets contained mustard oil, or peanut, sesame or coconut oils.

MATERIALS AND METHODS

Hearts from medico-legal autopsies which presented a broad cross-section of the population under study were examined. Brief information on the socioeconomic status, dietary habits and the nutritional status of deceased individuals was obtained. Heart specimens were obtained from 3 centers—Calcutta, Madras and Trivandrum, where the edible oils consumed differ. Samples obtained from Calcutta served as the study material since mustard oil is used in the diets there. The edible oil of preference in Trivandrum is coconut oil, whereas people in Madras use either peanut or sesame oils. Madras and Trivandrum were therefore included in the study as 2 important sources of control material for comparison with the study material from Calcutta.

The majority of the specimens from all 3 areas were from subjects in the age range 21-40 years. This was arranged deliberately since we thought this would have provided sufficient time for any effects of consumption of mustard oil to be manifest.

After the gross examination of the heart, it was fixed in 10% buffered neutral formalin. A 2-cm thick ring of the heart cut across the 2 ventricles midway between the apex and the atrioventricular rings was used for analyses. The tissue was washed free of formalin, minced and lipids extracted according to Folch et al. (8). The effect of storing the heart in formalin on the fatty acid profile of the lipid was investigated in a preliminary study and it was found that there was no difference in the fatty acid profile of heart lipid extracted either from fresh tissue or from tissue stored in formalin. However, some lipid did leach out during formalin fixation and the total lipid content therefore was not estimated. Methyl esters of fatty acids were prepared according to Fried (9). Analysis was done on a gas chromatograph with a 6-ft column of 15% diethyleneglycol succinate on Chromosorb W, with a flow rate of 30 ml N_2/min. The temperature was maintained between 198-200 C for column, injection port and flame ionization detector.

RESULTS

The fatty acid composition, expressed as the percentage of total fatty acids of the myocardial lipids from the 3 regions, are depicted in Table I.

Among the saturated fatty acids, lauric acid was found in the myocardium of subjects from Trivandrum only (3.5%). Similarly, heptadecanoic acid was only in the myocardium of subjects from Madras (1.4%). Arachidic acid was only in the Calcutta samples (1.9%). Palmitic acid was present to the same extent in the fat samples of hearts from all 3 centers (30.1, 26.3 and 31.0% in Madras, Calcutta and Trivandrum, respectively). The mean concentration of myristic acid was 3.0% in Calcutta samples, 7.3% in Trivandrum samples and 4.2% in Madras samples. The concentration of stearic acid was 8.4% in Madras samples, 6.7% in Calcutta samples and 4.9% in Trivandrum samples.

The concentration of oleic acid was higher than that of other unsaturated fatty acids in the myocardia received from all 3 centers. Its concentration was 39.1% in the lipid obtained from the hearts received from Madras, 31.0% in the myocardial lipid of samples received from Trivandrum and 35.0% in that of samples received from Calcutta. The concentration of palmitoleic acid was 13.3% in the myocardial lipid from Trivandrum, 9.3% in Calcutta samples and 8.4% in the lipids of hearts from Madras. The mean concentrations of linoleic acid in the myocardial lipids from Calcutta, Madras and Trivandrum were 12.2, 8.4 and 9.0%, respectively.

Erucic acid was present only in the samples received from Calcutta. Every heart had it, although the concentration varied widely from a low of 1% to a high of 9.9% with a mean of 5.6%. Not a single heart from Madras or from Trivandrum had even trace amounts of this fatty acid.

DISCUSSION

Although there are several reports to indicate the deleterious effects of consuming high levels of rapeseed oil and mustard oil in experimental animals, so far there has been no report to suggest its toxicity in human populations consuming rapeseed or mustard oil. There are no reports suggesting a higher incidence of cardiac abnormalities in areas in India where mustard oil is traditionally consumed compared to the incidence in areas where mustard oil is not consumed. As mentioned earlier, histopathological examination showed no difference in the incidence of fibrosis in the myocardia among the 3 centers.

Results of diet surveys carried out in several parts of Calcutta have shown that mustard oil provides between 1 and 3% of total daily calorie intake (6). Although monkeys fed a diet which contained 20% mustard oil developed myocardial fibrosis (5), those fed a diet which contained lower levels of either 5 or 10% mustard oil did not show fibrotic changes even after prolonged feeding (10), indicating that the level of mustard oil consumed was important in the development of myocardial fibrosis. In these studies on monkeys, the concentration of erucic acid in the myocardium was proportional to the level of mustard oil in the diet (10,11). The mean concentration of erucic acid in the myocardial fat of monkeys which had received 5 and 10% mustard oil were 9.5 and 14.4%, respectively, of the total fatty acids (10). In animals that received mustard oil at a 20% level, the concentration of erucic acid in the fat of hearts which showed fibrosis was

TABLE I

Profile of Major Fatty Acids of Human Myocardial Fat

Fatty acid		Calcutta	Madras	Trivandrum
Lauric	12:0	Trace	Trace	3.5 (0.7-12.0)
Myristic	14:0	3.0 (1.4- 7.1)	4.2 (2.2-10.8)	7.3 (3.6-12.9)
Palmitic	16:0	26.3 (18.7-36.7)	30.1 (17.4-52.4)	31.0 (19.8-43.7)
Palmitoleic	16:1	9.3 (1.8-19.7)	8.4 (2.1-17.4)	13.3 (7.2-22.5)
Heptadecanoic	17:0	ND[a]	1.4 (0.6- 6.7)	ND
Stearic	18:0	6.7 (2.7-14.1)	8.4 (1.8-21.4)	4.9 (0.8-11.7)
Oleic	18:1	35.0 (11.7-49.0)	39.1 (21.0-58.0)	31.0 (24.1-44.8)
Linoleic	18:2	12.2 (1.0-33.9)	8.4 (1.0-21.6)	9.0 (2.1-18.2)
Arachidic	20:0	1.9 (0.5-18.5)	Trace	ND
Erucic	22:1	5.6 (1.0-9.9)	ND	ND

Values expressed as percent of total fatty acids: mean and range of 50 samples from each region.

[a]ND = not detected.

38% of total fatty acids and that in non-fibrotic hearts was 24% of the total fatty acids (11). In the myocardial lipid of hearts received from Calcutta, the mean concentration of erucic acid was 5.6% of the total fatty acids, a level which was even lower than the mean concentration seen in monkeys fed 5% mustard oil. Even the highest concentration of erucic acid (9.9% of the total fatty acids) in the hearts received from Calcutta was well below the 14% seen in monkeys fed 10% mustard oil and somewhat similar to the level seen in the monkeys which had received 5% mustard oil. The concentration of erucic acid in the myocardial lipid in the human heart apparently fits in well with the level of mustard oil consumed in the diet. It is thus apparent that, at the level of intake of mustard oil prevalent in this country, the risk of developing myocardial fibrosis is small. However, whether the reduction in the mitochondrial respiration observed in monkeys fed 5 and 10% mustard oil (10) also can occur in this population and the significance of such a reduction are unclear. This aspect needs further investigation.

It is intriguing that the linoleic acid content of the myocardial lipid of samples received from Trivandrum were similar to those of the other 2 centers, in spite of the fact that coconut oil is a poor source of linoleic acid. From the information obtained on the dietary habits of this population, it was observed that fish is an integral part of the diet. Thus, it can be assumed that this essential fatty acid is integrated into the myocardiums of the Trivandrum population through the consumption of fish.

Long chain polyunsaturated fatty acids, especially arachidonic acid, which normally are present in the myocardial lipid (10), were not detected in the lipids of myocardium received from any of the centers. This probably is because there was a time lapse between death and autopsy; these polyunsaturated fatty acids might have degraded during this time.

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LETTER TO THE EDITOR

A Test for the Biochemical Efficiency of Isotopic Cholesterol

Sir:

The impurity of labeled compounds and their instability during storage has been the subject of many laboratory investigations. Breakdown of radioactive cholesterol, in particular, has been reported from at least 2 laboratories (1,2). Since isotopically labeled cholesterol is widely used in many biochemical as well as clinical studies, the purity of these preparations is of considerable importance. Snyder and Piantadosi (3) have emphasized the careful distinction between chemical and radiochemical purity, but Davidson et al. (4) found that, while certain tritiated cholesterol preparations were acceptable by chemical and radiochemical in vitro criteria, the same preparations were radiochemically unreliable in vivo during experiments with human subjects.

We have found that a tritiated cholesterol preparation (95% radiochemically pure by thin layer chromatography [TLC]) was only 60% efficient as a substrate for the enzyme lecithin: cholesterol acyltransferase (LCAT). Consequently, this enzymatic assay might be used in deciding whether a certain labeled cholesterol preparation is suitable or not for biochemical and in vivo experimentation.

In this communication, a simple method is described for the performance of such a test. The procedure is based on the comparison of serum cholesterol esterification measured by isotopic and chemical methods. The assay system using labeled cholesterol has been described (5) and it is based on the original technique of Stokke and Norum (6). Briefly, fresh human serum is incubated with 5,5'-dithiobis (2-nitro-benzoic acid; DTNB) for 30 min at 37 C to temporarily inactivate the enzyme. Labeled cholesterol is added next as a human serum albumin emulsion (6) and allowed to incubate with the serum for 4 hr at 37 C to facilitate the transfer of [^{3}H]-cholesterol from the albumin to the lipoproteins. Aliquots are removed at the selected time intervals (including 0 time) into chloroform/methanol (2:1) for lipid extraction. The extracts are then concentrated and TLC, staining and scintillation counting are performed as described previously (7). The percentage esterification for each time period is calculated upon counting the labeled free and esterified cholesterol. The radioactive measurements are then compared to the amount of cholesterol esterified as determined by gas chromatography (GC) (6,8) or by a colorimetric method (9,10). The labeled cholesterol may be judged of good quality if the results with radioactive substrate are 95% or higher of those obtained by GC or colorimetry.

We have used this assay system in our laboratory for several years for the analysis of hundreds of serum samples with excellent reliability. Upon switching from one radioactive cholesterol preparation to another, however, the activity of the control dropped 40% (Table I). This determination was repeated more than 20 times and always gave the same low values. The reduced substrate efficiency was reflected in the comparison of the esterification measured by radioactive counting to chemical determination (10). The radioactive counting turned out to be ca. 50% of the chemical determination (Table II).

Upon careful examination of each component of the assay mix (phosphate buffer, DTNB, serum albumin preparations and mercaptoethanol), we concluded that only the labeled cholesterol (Schwarz/Mann prep #2) could be responsible for the unusually low results obtained. We paid particular attention to eliminating the possibility that inadequate equilibration of the labeled cholesterol with lipoprotein cholesterol caused the change in cholesterol esterification (11). Since the procedure for the introduction of labeled cholesterol was identical for all assays (4,5) and only one of the isotopic samples gave substantially and repeatedly lower results, we concluded that insufficient equilibration of the labeled and unlabeled substrate could not be the cause of our findings. Furthermore, Wallentin and Vikrot have shown (12) that the 4-hr incubation period with labeled cholesterol (recommended by Stokke and Norum [6] and used in our procedure) is more than sufficient to achieve complete equilibration with the lipoprotein cholesterol pool.

The most feasible explanation of our findings is that the major contaminant present in the low efficiency (Schwarz/Mann prep #2)

TABLE I

Measurement of the Initial Rate of Serum Cholesterol Esterification Using Various Tritiated Cholesterol Preparations

Source of labeled cholesterol	Schwarz/Mann		New England Nuclear
	Prep #1	Prep #2	Prep #1
Cholesterol esterified μmol/ml/min	1.3	.765	1.33

TABLE II

Comparison of Values Obtained for Serum Cholesterol Esterification Measured by Colorimetric Assay (10) and Radio Assay (5)[a]

Source of labeled cholesterol		Schwarz/Mann		New England Nuclear
		Prep #1	Prep #2	Prep #1
Cholesterol esterified	1[b]	342	342	342
mμmol/ml	2[b]	328	188	331
Efficiency of radioactive cholesterol preparation as indicated by the assay		96%	55%	97%

[a]Incubation for 8 hr at 37 C.

[b]1: Colorimetric assays; 2: radio assay.

preparation, is a compound similar to cholesterol to the extent that it travels with cholesterol on TLC while it is sufficiently different to be rejected by lecithin:cholesterol acyltransferase for esterification.

The fact that LCAT was unable to use this sample of tritiated cholesterol (Schwarz/Mann prep #2) fully, raises the possibility that such a preparation would not react normally in other biological systems.

Contaminated and deteriorated isotopic compounds may turn up in any laboratory. The presence of a contaminant in a tritiated cholesterol preparation already has been reported by Bayly and Evans (1) who found that even reverse isotope dilution analysis failed to detect the presence of the impurity. Further, Davidson et al. (4) recently have found that all in vitro criteria failed to indicate any contamination in tritiated cholesterol preparations which, nevertheless, behaved anomalously upon injection into human subjects. Davidson et al. recommend that all batches of radioactive cholesterol be tested in an in vivo assay system before they are used in metabolic experiments.

The method described in this communication could well serve to test the biological efficiency of labeled cholesterol samples without involving more costly and complicated in vivo techniques. The means of testing the biochemical efficiency of labeled cholesterol thus should be useful to all investigators who routinely use these isotopic preparations.

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Sterol Analysis of the Inner and Outer Mitochondrial Membranes in Yeast

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ABSTRACT

The membranes of yeast mitochondria were separated and analyzed for lipid content. The sterol-to-phospholipid molar ratio was found to be very similar between the inner and outer membranes (1:30). These observed ratios could be substantially altered by using a crude mitochondrial pellet contaminated with a "floating lipid layer." In this case, the sterol-to-phospholipid molar ratios were 1:8 to 1:26 for the outer and inner mitochondrial membranes, respectively.

INTRODUCTION

Sterols play a prominent role in the fluidity, integrity and structure of eucaryotic membranes. Thus, in organelles such as mitochondria, where membranes control the function of the organelle, it is essential to know how the sterol is distributed. Toward this end, mitochondrial membranes from several systems have been analyzed for their lipid content (1-5). Thus far, no sterol analysis of the inner and outer mitochondrial membranes has been reported for yeast. However, a study with another fungus, *Neurospora crassa,* concluded that sterols were located exclusively in the outer mitochondrial membrane (3). This was inconsistent with our previous experiments in which sterol modifications effected alterations in the Arrhenius plots of the inner mitochondrial membrane enzyme cytochrome oxidase (6). In order to resolve this apparent discrepancy and to ascertain the importance of sterol in yeast mitochondria, we have separated the mitochondrial membranes in *Saccharomyces cerevisiae* and determined the amount of ergosterol present in each membrane with respect to other membrane components.

METHODS AND MATERIALS

Culture Conditions

An isolate of MCC, a wild-type diploid strain of *Saccharomyces cerevisiae,* was grown to late exponential phase (20 hr) at 28 C with aeration in media containing tryptone (1%), yeast extract (0.5%) and ethanol (2% v/v).

Isolation of Mitochondria

Cells were harvested by centrifugation at 5,000 G for 1 min and washed with distilled water (10 ℓ of media typically yielded 60 g wet wt of cells.) The cells were resuspended in 0.5 M β-mercaptoethanol, 0.1 M Tris buffer, pH 9.3, at a ratio of 2 ml buffer/g wet wt of cells and incubated at 28 C with gentle shaking for 5 min. The cells were pelleted (12,000 G, 1 min) and washed twice with a buffer solution at pH 5.8 containing 0.7 M sorbitol, 0.3 M mannitol, 0.1 M citrate, 10 mM K_2HPO_4, and 1 mM EDTA. To form spheroplasts, the washed pellet was resuspended in the same buffer containing 20% Glusulase, an enzyme extract from *Helix pomatia,* at 2.5 ml buffer/g wet wt cells. The suspension was incubated for 1 hr at 28 C with gentle shaking. The spheroplasts were pelleted by centrifugation at 3,000 G for 10 min, washed 3 times in a buffer containing 0.9 M sorbitol, 10 mM Tris, and 0.5 mM EDTA at pH 7.4, and then resuspended in the same buffer at 10 ml/6 g wet wt spheroplasts. The spheroplast suspension was passed through a French pressure cell at 1,000-2,000 psi. Unbroken spheroplasts and cell debris were removed by 3 centrifugations each at 1,100 G for 10 min. To pellet the mitochondria, the supernatant was spun at 12,000 G for 20 min. The mitochondria were resuspended in the above 0.9 M sorbitol buffer and centrifuged at 1,100 G for 10 min to remove the last of the cellular debris. The supernatant was centrifuged once again at 12,000 G for 20 min to recover a crude mitochondrial pellet. This mitochondrial pellet was resuspended in the same buffer, placed on 20-70% linear sucrose gradients and centrifuged for 30 min at 22,700 G in a Sorvall Model SS90 vertical rotor. The gradients were fractionated and each fraction assayed for marker enzymes (see Results). The major band was identified as mitochondrial and was repelleted by centrifugation at 27,000 G for 20 min.

Separation and Isolation of the Inner and Outer Mitochondrial Membranes

In a procedure modified from Neupert and Ludwig (7), the mitochondria were subjected to osmotic swelling and shrinking to enhance the membrane separation, followed by our pro-

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cedure of mechanical shearing through an Eaton pressure cell to strip the outer mitochondrial membrane away from the inner membrane envelope. The whole mitochondria were swollen by resuspending the mitochondrial pellet in 20 mM Tris buffer at pH 8.0 (1 ml buffer/gm wet wt mitochondria) and incubating on ice for 15 min. A solution of 1.8 M sorbitol, 2 mM ATP, 2 mM $MgCl_2$ was then added (0.5 ml sorbitol-ATP-$MgCl_2$ solution/g wet wt mitochondria) and the suspension was left on ice for 1 hr to allow shrinkage of the inner membrane. The mitochondrial suspension was quickly frozen in an Eaton pressure cell and, while still frozen, passed through the small orifice of the cell at 5000 psi. The sheared mitochondria were diluted by adding 2 ml of 20 mM Tris buffer at pH 8.0/g wet wt mitochondria, loaded on 10-20-30-40-50-70% (1:2:2.5:2.5:2.5:1 vol ratios) discontinuous sucrose gradients, and centrifuged at 50,000 G for 1.5 hr. The gradients were fractionated in 1-ml vol from the top and each faction was analyzed for total free ergosterol, phospholipid, protein and enzyme activity.

Quantitation of Sterol, Phospholipid and Protein

The lipids were extracted from each fraction and from whole mitochondria by the Bligh and Dyer method (8) and separated by thin layer chromatography (TLC) using the Skipski et al. procedure (9). Ergosterol was quantitated by gas liquid chromatography (GLC) using cholestane as an internal standard (10). Cholesterol was added during the extraction procedure as a carrier sterol. The quantitation of the recovered cholesterol by GLC also provided a measurement of the efficiency of the sterol extraction.

Phospholipid samples were taken from the lipid extracts described prior to the TLC separation, and were quantitated by the Ames method (11). Protein was determined for each fraction by the Lowry et al. procedure (12). Bovine serum albumin (BSA) was used as the protein standard.

Enzyme Markers

The following enzymes have been localized by Bandlow and Bauer (13) in yeast and were used as marker enzymes to determine the composition of each fraction and the degree of cross-contamination. Rotenone-insensitive NADH-cytochrome c reductase (outer mitochondrial membrane), succinate-cytochrome c reductase (inner mitochondrial membrane) and NADPH-cytochrome c reductase (microsomal) were assayed by the Sottocasa et al. methods (14). Malate dehydrogenase activity (mitochondrial matrix) was measured as described by Vary et al. (15), cytochrome c oxidase activity (inner mitochondrial membrane) as described by Thompson and Parks (16), and kyurenine hydroxylase activity (outer mitochondrial membrane) as described by Schott et al. (17). Chitin synthetase, identified as a plasma membrane marker in yeast (18), was assayed by Cabib's method (19).

Mitochondrial Respiration

Mitochondrial respiration was measured at 28 C in an oxygraph buffer at pH 6.6 containing 15 mM Tris, 15 mM maleate, 0.67 ml phosphoric acid, 1.2 mM EDTA, 6 mM $MgCl_2$ and 0.2% BSA. Ethanol was used as the respiratory substrate.

Materials

Glusulase was purchased from Endo Laboratories. All enzyme assay reagents, L-kyurenine, rotenone, cytochrome c, oxaloacetate, NADPH, BSA, NADH, trypsin, trypsin inhibitor, N-acetylglucosamine and succinate were from Sigma Chemical Co., St. Louis, Mo. UDP-[^{14}C]-N-acetylglucosamine was purchased from New England Nuclear, Boston, MA.

Instrumentation

A Beckman L-65 ultracentrifuge and Sorvall RC-2B centrifuge were used for the centrifugations. The Eaton pressure cell (20) was used in a Carver Model B laboratory press. The enzyme assays were conducted with a PMQ II Zeiss spectrophotometer and the mitochondrial respiration measured on a Gilson oxygraph equipped with a Clark O_2 electrode. GLC separations of sterols were performed with a Varian Series 2700 gas chromatograph equipped with a CDS-111 data processor and a Supelco SP-2250 column.

RESULTS

Assessment of the Purity of Membrane Fractions

The purity and intactness of the mitochondrial preparation is established in Table I. NADPH-cytochrome c reductase and chitin synthetase specific activities (sp act) showed that there was little contamination of the mitochondria with microsomal or plasma membrane protein. The mitochondria also appeared to be intact, exhibiting respiratory competency (RC values of at least 2.5, as calculated from state 3/state 4) and little mitochondrial enzyme release. This fact was important in that mitochondria which were prepared from mechanically broken cells (i.e., shaking with glass beads or disruption in a Bronwill MSK tissue homogenizer) were damaged and thus did not swell or shrink when placed in the anisotonic buffers.

TABLE I

Assessment of the Purity and Intactness of the Mitochondria

Fraction	Malate dehydrogenase (μmol NADH oxidized)	NADPH-Cyt C Reductase (μmol cyt c reduced)	Chitin synthetase (microunits)
	min-mg protein	min-mg protein	mg protein
12,000 G supernatant (microsomes and cytosol)	9.2	24.2	56.1
12,000 G pellet (crude mitochondria)	105.1	0.6	24.5
Major gradient band (purified mitochondria)	168.2	0.0	1.5

Malate dehydrogenase (mitochondrial matrix enzyme) specific activities were calculated from the extinction coefficient of NADH (ϵ = 6.22 x 10^3 $M^{-1}cm^{-1}$) at 340 nm. NADPH-cytochrome c reductase (microsomal enzyme) specific activities were calculated from the extinction coefficient of cytochrome c (ϵ = 19.1 x 10^3 $M^{-1}cm^{-1}$) at 550 nm. One unit of chitin synthetase (plasma membrane enzyme) activity is the incorporation of 1 μmol of acetylglucosamine/min into chitin (19). Enzyme activities were measured as described in Methods and Materials.

Since the swelling and shrinking of the mitochondrial membranes proved necessary for adequate separation, the organelle had to be intact prior to treatment.

Inner and outer mitochondrial membranes from gradient-purified mitochondria could be satisfactorily separated after passage through the Eaton press as demonstrated by the membrane marker enzymes (Table II). The distribution of marker enzyme activities reveal minor cross-contamination of these membranes (Fig. 1). This cross-contamination was on the order of 10%—apparently typical in such separation experiments (7,21,22). Despite repeated attempts, the cross-contamination could not be reduced and other methods of disruption, e.g., sonication (7), homogenization (23) and treatment with ionophors (13), failed to isolate the inner membrane as cleanly.

The gradient fractions containing membrane were identified on the basis of the enzyme markers (Table II). Fraction 1 (0-10% sucrose) had neither inner nor outer mitochondrial membrane enzyme activities. It did, however, contain some protein. Fractions 2-3 (10-20% sucrose) contained the outer mitochondrial membrane. Fractions 4, 5 and 6 (20-30% sucrose) had essentially no assayable enzyme activity but did contain protein. Fractions 7-8 (30-40% sucrose) had both outer and inner mitochondrial membrane enzyme activity and probably represented the formation of mixed vesicles. Compared to the total enzyme yield, however, this fraction was small. The inner mitochondrial membrane was in fraction 9-10 (40-50% sucrose). The density of the inner

TABLE II

Total Activities of Marker Enzymes in Gradient Fractions

Fraction	Cytochrome oxidase (μmol cyt ox)	Succinate-cyt C reductase (μmol cyt red)	NADH-cyt C reductase (μmol cyt red)	Kyurenine hydroxylase (nmol hydrox)
	min	min	min	min
1	1.8	0.5	5.5	0.1
2-3	11.2	1.3	90.6	4.7
4-6	7.3	1.2	6.3	0.2
7-8	19.6	3.3	19.1	0.8
9-10	53.4	7.3	11.8	0.7
11-12	7.3	0.8	1.1	0.2

The inner and outer membranes of gradient-purified mitochondria were separated and identified by marker enzymes. Cytochrome oxidase (inner membrane enzyme), succinate-cytochrome c reductase (inner membrane enzyme), and rotenone-insensitive NADH-cytochrome c reductase (outer membrane enzyme) total activities were calculated on the basis of the cytochromic c extinction coefficient (ϵ = 19.1 x 10^3 $M^{-1}cm^{-1}$) at 550 nm. Kyurenine hydroxylase (outer membrane enzyme) total activities were calculated on the basis that 20 μg hydroxylated kyurenine has an optical density 0.183 units at 492 nm (17). Enzyme assays were conducted as described in Methods and Materials.

mitochondrial membrane was just slightly higher than whole mitochondria run on the same gradients. Fractions 11-12 (50-70% sucrose) had little enzyme activity or protein.

Quantitation of Sterol, Phospholipid and Protein in Membrane Fractions

The analysis of the sterol, phospholipid and protein in each fraction is provided in Table III. The ergosterol-to-phospholipid molar ratio of the gradient-purified whole mitochondria was 1:33. The inner (fractions 9-10) and outer (fractions 2-3) mitochondrial membranes contained similar ergosterol-to-phospholipid molar ratios of 1:30 and 1:29, respectively.

The ratio of ergosterol to protein in whole mitochondria was similar to the inner mitochondrial membrane. The outer membrane, though, had a higher ratio of ergosterol to protein than the inner membrane, as expected since the inner membrane is known to contain more protein than the outer membrane (24).

If the crude mitochondrial pellet (i.e., the mitochondrial pellet prior to gradient purification) was subjected to the separation procedure, a different pattern of sterol ratios was observed as outlined in Table IV. (Note: The crude mitochondria did have the same marker enzyme distribution [Fig. 1] as the gradient-purified mitochondria separations). The ergosterol-to-phospholipid molar ratio of the whole crude mitochondria was significantly higher (1:14) than the gradient-purified mitochondria. The inner and outer membranes also had slightly higher ratios of 1:26 and 1:22. However, the major difference was the appearance of a "floating lipid layer" (fraction 1), which was unobserved in the gradient-purified mitochondria separations. This "floating lipid layer" had extremely high molar ratios of ergosterol to phospholipid (1:2) and ergosterol to protein (0.055 umol/mg), and thus represented a unique fraction.

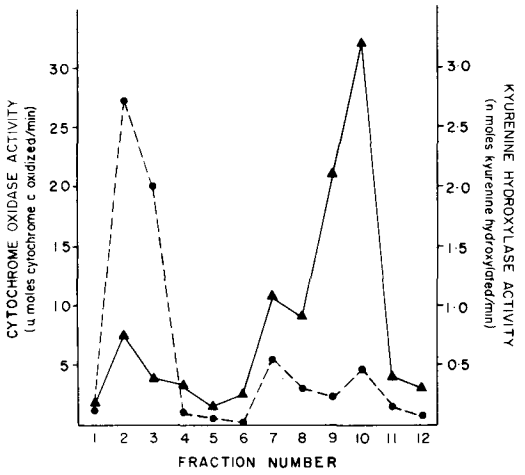

FIG. 1. Total activities of marker enzymes vs gradient fractions. The inner and outer membranes of gradient-purified mitochondria were separated on discontinuous sucrose gradients and identified by marker enzymes. Cytochrome oxidase (—▲—▲—▲—), the inner membrane marker, total activities were calculated from the extinction coefficient of cytochrome c (ϵ = 19.1 x 10^3 M^{-1} cm^{-1}) at 550 nm. Kyurenine hydroxylase (--●--●--●--), the outer membrane marker, total activities were calculated on the basis that 20 μg hydroxylated kyurenine has an optical density of 0.183 units at 492 nm (17). Fraction numbers increase with the density of the gradient, fraction 1 being the top of the gradient. Each fraction represents a 1 ml volume. Preparation of the gradients and enzyme assays are described in Methods and Materials.

DISCUSSION

Our results demonstrate that the ratios of free sterol to the other membrane components

TABLE III

Lipid analysis of the Fractions Derived from Gradient-Purified Mitochondria

Fraction	Sterol/protein (μmol/mg)	Phospholipid/protein (μmol/mg)	Sterol/phospholipid (μmol/μmol)
1	0.0008 ± 0.0001	0.157 ± 0.001	0.0051
2-3 (outer)	0.0142 ± 0.0002	0.416 ± 0.019	0.0341
7-8 (mixed)	0.0094 ± 0.0003	0.232 ± 0.004	0.0405
9-10 (inner)	0.0088 ± 0.0001	0.266 ± 0.008	0.0331
Total	0.0098 ± 0.0005	0.311 ± 0.007	0.0315
Whole mitochondria	0.0094 ± 0.0001	0.313 ± 0.007	0.0300

The mitochondria used for these membrane separations were gradient-purified as outlined in Methods and Materials. The quantitation of the sterol, phospholipid and protein is described in Methods and Materials. All values are expressed as total μmol or mg/gradient graction. The fractions containing membrane were identified on the basis of marker enzyme activities (see text for description). Each entry is the average of 5 experiments and is uncorrected for cross-contamination.

TABLE IV

Lipid Analysis of the Fractions Derived from Crude Mitochondria

Fraction	Sterol/protein (μmol/mg)	Phospholipid/protein (μmol/mg)	Sterol/phospholipid (μmol/μmol)
1 (lipid layer)	0.0546 ± 0.0014	0.108 ± 0.016	0.5056
2-3 (outer)	0.0180 ± 0.0026	0.399 ± 0.020	0.0451
7-8 (mixed)	0.0186 ± 0.0031	0.375 ± 0.019	0.0497
9-10 (inner)	0.0101 ± 0.0012	0.263 ± 0.019	0.0384
Total	0.0233 ± 0.0031	0.275 ± 0.020	0.0847
Whole mitochondria	0.0252 ± 0.0030	0.344 ± 0.031	0.0738

The mitochondria used for these membrane separations were the crude mitochondrial pellets taken prior to gradient purification as described in Methods and Materials. The sterol, phospholipid and protein were quantitated as detailed in Methods and Materials. All values are expressed as total μmol or mg/gradient fraction. The fractions containing membrane were identified on the basis of marker enzyme activities (see text for description). Each entry is the average of 5 experiments and is uncorrected for cross-contamination.

TABLE V

Comparison of Mitochondrial Sterol Ratios from Different Systems

Source	Sterol/phospholipid (μmol/μmol)			Sterol/protein (μmol/mg)			Ref.
	Whole mitos	Outer membrane	Inner membrane	Whole mitos	Outer membrane	Inner membrane	
Rat liver	1:10	1:8	<1:100	0.0160	0.0552	<0.0015	(1)
Rat liver	1:10	1:4	1:29	0.0210	0.0559	0.0087	(25)
Pig heart	1:24	1:15	1:91	0.0120	0.0260	0.0047	(5)
Guinea pig heart	1:39	1:16	1:33	0.0059	0.0778	0.0131	(4)
Neurospora	1:11	1:3	1:32	0.0378	0.4836	0.0126	(3)
Yeast							
Crude	1:14	1:8[a]	1:26	0.0252	0.0355[a]	0.0101	this paper
Purified	1:33	1:29	1:30	0.0094	0.0142	0.0088	this paper

[a]Ratios of the "floating lipid layer" (fraction 1) is included as part of the outer membrane (fractions 2-3).

Mitochondrial sterol ratios from different systems were compiled directly from values reported in the corresponding publication or calculated from the given data. The mitochondrial sterol ratios for yeast were calculated from Tables II and III. Ergosterol: MW = 397; cholesterol: MW = 387; average phospholipid: MW = 700.

do not vary significantly between the inner and outer mitochondrial membranes in yeast. This is incongruous with previous work in rat liver (1,25), guinea pig heart (4), pig heart (5) and *N. crassa* (3) mitochondria, where the sterol-to-phospholipid molar ratios in the outer membranes ranged from 1:3 to 1:16 and the inner membranes ranged from 1:29 to 1:100 (Table V). In these publications, it was concluded that the mitochondrial membranes were very different in sterol composition.

Yeast, then, are distinct from other systems where the outer mitochondrial membrane have been reported to have higher sterol ratios than the inner membrane. Our precise separation and quantitation of sterols, and effective isolation and fractionation of yeast mitochondria support this conclusion. The basis for this difference is the sterol ratios of the outer membrane of yeast mitochondria. Both the ratios of sterol to phospholipid and sterol to protein for the yeast outer membrane are substantially lower than any other published report (Table V). The inner membrane and whole mitochondria of yeast, on the other hand, are comparable to several other systems (Table V). The yeast whole mitochondria, e.g., have sterol ratios similar to pig heart and guinea pig heart mitochondria. The inner membrane of yeast mitochondria contained a significant amount of sterol like many other reports. In some cases (1,5), though, the inner membrane was reported to be free of sterol.

The disparity between previous reports and our present work may be explained on the basis of differences in experimental methods. Reported sterol-to-phospholipid molar ratios can vary widely, even in whole mitochondria from

the same source (e.g., rat liver mitochondria [1,26]). Quantitative techniques for the lipids, particularly the sterols, may be a major cause of these variations. As the amount of sterol esters in the purified yeast mitochondria was extremely small (data not given), sterol esters and precursors were not included in our data as they were in those reports where the Libermann-Burchard assay for sterols or saponification extraction for lipids was employed. Only the "floating lipid layer" from the crude mitochondria showed demonstrable quantities of sterol esters. This contaminating lipid source was eliminated in our preparations.

Another possible explanation as to the disagreement between other reports and our results may lie in the purity of the mitochondria. In our experiments with yeast, gradient-purified mitochondria did not have the "floating lipid layer," which suggests that it is a lipid-rich membrane contaminating crude mitochondrial preparations. In light of the relatively high activity of the plasma membrane marker, chitin synthetase, in the crude mitochondrial pellet, this contaminating lipid may be small plasma membrane vesicles. Since this "floating lipid layer" has a high sterol-to-phospholipid molar ratio, inclusion of this fraction as part of the outer mitochondrial membrane by experimentors would produce a large difference between the sterol ratios of the inner and outer membranes. In particular, this would explain the discrepancy between our results for yeast and those reported for *Neurospora*.

Although some reports indicated that essentially no sterol is present in the inner mitochondrial membrane (1,3,5), we find sterol associated with this membrane. In view of various experiments, including our own, that demonstrated the influence of sterols on inner mitochondrial membrane enzymes (6,25,27), it should be no surprise that free sterols are part of that membrane. Since sterols provide both fluidity and rigidity to membranes, their presence may be necessary in the inner mitochondrial membrane to insure the enzymes a suitable environment in which to function (28).

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Fatty Acid Synthesis in vivo and Hepatic Contribution to Whole-body Lipogenic Rates in Obese Zucker Rats[1]

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ABSTRACT

We have re-examined the claim by Godbole and York, based on the effect of surgical hepatectomy (Diabetologia 14:191, 1978), that liver contributed more than 90% of the newly synthesized FA found in adipose tissue of obese rats at the end of a 1-hr pulse of ^3H_2O. The amount of newly synthesized FA transported via plasma VLDL from liver to adipose tissue was estimated in lean and obese Zucker rats by determining the effects of Triton WR-1339, which blocks the uptake of VLDL-TGFA into tissues. Triton treatment was found not to cause any significant change in the amount of radioactive FA found in subcutaneous/perimetrial fat tissues, carcass or liver in either chow-fed or high-glucose, fed-refed lean or obese rats, although in the fed-refed dietary state the proportion found in the liver was increased over that in the chow-fed groups. Furthermore, the amounts of newly made FA which accumulated in the plasma of Triton-treated, chow-fed and glucose-fed refed animals during this period constituted only a few percentages of those found in the adipose tissue of these animals. Thus, in contrast to the claims of Godbole and York, no significant transfer of newly made FA from liver to adipose tissue occurs during a 1-hr experiment; it follows that the amounts of these FA found in different tissues at the end of that period are valid measurements of their actual lipogenic activities in situ. It is suggested that the Godbole and York results are artifacts of their surgical hepatectomy procedure.

ABBREVIATIONS

TG, triglycerides; VLDL, very low density lipoprotein(s); FA, fatty acids; TLFA, total lipid fatty acids.

INTRODUCTION

The genetically obese rat deposits excessive quantities of fat in its adipose tissues (1). Many studies have shown that both hepatic and extrahepatic FA synthesis, as well as hepatic production of VLDL-TG, are increased in obese as compared to lean Zucker rats (2-4), but the relative importance of the liver and adipose tissue in the synthesis of the excess lipids deposited in the lean rats is still contested. In a recent paper (5), Godbole and York made very strong claims for the dominant role of the liver in this process, based primarily on their observation that hepatectomy decreased by 10-fold the amount of newly synthesized FA which was found in the major adipose tissues of obese Zucker rats 1 hr following intravenous injection of ^3H_2O. However, since this decrease could be secondary to the severe upset of normal metabolism produced by this traumatic procedure, and since the rates of appearance of serum ^{3}H-TGFA reported by these authors were inconsistent with their own interpretation, we have re-examined this question, using Triton WR-1339 (6), rather than surgical hepatectomy, to block any transfer of newly synthesized FA from the liver to adipose tissue during the experiment; conditions were otherwise similar to those of Godbole and York (5). We also extended the same approach to animals on a fat-free, high-glucose diet, which we have shown to induce maximal hepatic lipogenesis in mice, with the liver accounting for over one-half of total body FA synthesis (7). Our results are straightforward: during the period of 1 hr used by Godbole and York, there is no detectable transfer of newly synthsized FA from the liver to adipose tissue; the amounts of these FA in different tissues at the end of that period therefore reflect their relative indigenous rates of FA synthesis.

EXPERIMENTAL PROCEDURES

Rats

Female obese Zucker fa/fa rats and their lean litter mates (+/?) were purchased from Byrd Memorial Laboratories, Boston, MA. Six-week-old rats with mean body weights of 118 g (lean) and 157 g (fa/fa), maintained on Purina chow ad libitum were used for the first experiment, and designated "chow-fed." Other 6-week-old rats were switched to a 58% glucose fat-free diet (8) 2 weeks prior to the second experiment and designated "high-glucose, fed-

[1]Presented in part at the 1979 meeting of the American Society of Biological Chemists.

refed group"; their mean body weights were 174 g (lean) and 239 g (fa/fa).

Injection of Triton WR-1339 and ^3H_2O

Triton WR-1339 (600 mg/kg, as a 20% w/v solution in saline) was injected into a tail vein of unanesthetized lean and obese rats (n = 4/group) held in restraining cages. This was followed within 2 min by ^3H_2O (200 μl, 2.5 mCi) intraperitoneally (ip). Control animals were given ^3H_2O only. The high-glucose, fed-refed group (4/group; lean and obese) received 1 g glucose/100 g body weight as a 50% aqueous glucose solution intragastrically 30 min prior to ^3H_2O injection.

Sampling of Tissues

All rats were killed by decapitation 60 min after ^3H_2O injection. Blood was collected in heparinized tubes; plasma was separated and stored frozen until analyzed. Livers were perfused with 100 ml saline, blotted on filter paper, weighed and frozen in liquid nitrogen. About 1 g (lean), or 3 g (obese) of subcutaneous and perimetrial fat tissues were dissected out, weighed and frozen. The remaining carcass was frozen in liquid nitrogen and stored frozen.

Analysis of Tissue Fatty Acids

Liver samples (2-3 g) were saponified with 10 ml of 15% KOH in 50% ethanol for 2 hr at 80 C. After extraction of nonsaponified lipids and acidification with 12 N HCl, TLFA were extracted with 15 ml petroleum ether (30-60 C), washed twice with 10 ml water, and an aliquot dried down and counted in a 1:1 Instagel-toluene mixture. Appropriate quench corrections were made with internal standards.

Subcutaneous and perimetrial fat tissues (1-2 g) were worked up in a similar manner, with frequent shaking during a 4-hr digestion with 15% KOH in 50% ethanol, to ensure complete saponification of the large amounts of triglycerides.

Carcasses of lean and obese rats were digested with 350 and 480 ml, respectively, of 30% KOH in 50% ethanol at 80 C for 2 hr. Extraction and counting procedures were the same as for liver. To correct for chemical exchange of solvent $^3H^+$, unlabeled carcasses of one lean and one obese rat were processed with addition of ^3H_2O to the saponification mixture. The corrections amounted to less than 10% of the experimental values.

Total lipids from blood plasma were extracted according to Bligh and Dyer (9). Fatty acids were obtained as already described after saponification in 1 ml 15% KOH in 50% ethanol. Plasma TG were estimated according to Galletti (10).

Plasma ^3H_2O specific activity (sp act) was determined at t = 60 min and assumed to equal that of body water (5). Rates of FA synthesis from ^3H_2O were calculated using the Windmueller and Spaeth formula (11).

RESULTS

The accumulation of newly synthesized fatty acids in perimetrial and subcutaneous adipose tissue, expressed as μmol/g tissue/hr and in total liver and extrahepatic carcass, in μmol/hr, are shown for chow-fed and high-glucose, fed-refed groups in Tables I and II, respectively. Table III summarizes the relative accumulation of newly made FA in total liver, plasma, extrahepatic carcass and a calculated "average" total adipose tissue (see following) in all experimental groups.

In agreement with the results of Godbole and York (5), the accumulation of newly made FA in total liver, and per g perimetrial and subcutaneous adipose tissue, were about one order of magnitude greater in obese compared to lean, chow-fed Zucker rats (Table I). Most significantly, however, the data in Table I further show that none of these rates were significantly affected by the administration of Triton WR-1339, the agent used to block the uptake of liver-derived FA by adipose tissues. In the obese group, the effectiveness of the Triton block was demonstrated by an increase in the levels of serum TG concentration from 167 ± 25 to 786 ± 96 mg/dl. However, the amounts of newly synthesized, i.e., labeled, FA trapped in serum lipids of these animals were low and not different from those found in the untreated controls; in fact, the radioactivity in the aliquots of serum TGFA taken in this experiment was too low to be accurately determined. These values are, therefore, given in Table III as estimated maximal values. Thus, during the brief 1-hr pulse experiment with tritiated water, which was also used by Godbole and York (5), we found neither measurable transfer of newly made FA from liver to adipose tissue, nor, indeed, significant release of such labeled FA from the liver into the circulation in either lean or obese Zucker rats.

The only statistically significant difference between Triton-treated animals and controls in Table I was a small decrease (18%) in the total amount of newly synthesized FA in the carcass of Triton-treated, lean rats; its metabolic significance seems doubtful. More to the point is a comparison between the rates of accumulation in adipose tissue and total carcass. The

TABLE I

Accumulation of Newly Synthesized Fatty Acids in Tissues of Chow-Fed, Lean and Obese Zucker Rats 1 Hr Following Injection of ^3H_2O[a]

Animals	Triton[b]	Newly synthesized fatty acids				Calculated average adipose tissue
		Liver	Carcass	Adipose tissue[c] SC	Adipose tissue[c] PM	
		μmol/1 hr		μmol/g·hr		μmol/1 hr
Lean	-	3.0 ± 0.7	33 ± 1	0.38 ± 0.18	0.34 ± 0.07	2
		ns[e]	<0.01	ns	ns	
	+	4.8 ± 0.3	28 ± 1	0.18 ± 0.06	0.28 ± 0.07	
Obese	-	27 ± 13	103 ± 2	1.6 ± 0.5	1.6 ± 0.8	110
		ns	ns	ns	ns	
	+	35 ± 5	117 ± 12	1.5 ± 0.7	3.2 ± 0.3	

[a]Mean ± SE from 4 rats/group; average body weights: lean 118 g, obese 157 g.

[b]Triton WR-1339 (200 mg/ml, 600 mg/kg BW) was injected iv into unanesthesized rats.

[c]SC: subcutaneous; PM: perimetrial.

[d]Using numerical average of μmol/g/hr for SC and PM in both Triton and non-Triton groups (see text).

[e]ns: not significant.

maximum calculated total adipose tissue in our lean and obese rats would be ca. 7 and 47 g, respectively (taking total body fat to be 5 and 25% of body weight in our lean and obese rats [see following] and to be entirely accounted for as adipose tissue containing 83% [w/w] fat [12]). Assuming this behaved as a simple average of the perimetrial and subcutaneous adipose tissues shown in Table I, this average total adipose tissue should accumulate ca. 2 μmol/hr of newly synthesized FA in lean rats, an order of magnitude less than the 33 μmol/hr actually found in the total extrahepatic carcass of these animals. Thus, under the conditions of our experiment, most extrahepatic FA synthesis in lean, chow-fed rats occurred either in adipose tissues much more active than the major perimetrial or subcutaneous depots, or in other, non-adipose tissues. In the obese animals, however, the calculated average accumulation of newly synthesized FA in adipose tissue would be ca. 110 μmol/hr, i.e., the same as the average actually found in extrahepatic carcass. Thus, in the obese animals, adipose tissues such as the perimetrial and subcutaneous depots were sufficient to account for extrahepatic accumulation of newly synthesized FA in the total carcass (Table III).

Table II summarizes the results obtained when the experiment just described was repeated on high-glucose, fed-refed animals. Compared to the chow-fed group, lean animals responded to this hyperlipogenic regimen with 5- to 7-fold increased rates of deposition of newly made FA in both liver and adipose tissues, which brought these rates up to the same level as those in chow-fed, obese animals (Table I). In the high-glucose, fed-refed obese animals, however, only the liver showed a marked increase (about 4-fold) compared to the chow-fed, obese group, whereas the rates per g perimetrial and subcutaneous adipose tissues remained essentially unchanged (compare Tables I and II). Again, Triton WR-1339 failed to produce any significant change in the accumulation of newly made FA in any of the tissues in either lean or obese animals. Further, although the Triton treatment increased the concentration of plasma TG 10-fold in both groups (from 35 ± 15 to 408 ± 17, and 45 ± 17 to 580 ± 300 mg/dl for lean and obese, respectively), the content of newly synthesized plasma TGFA, which were accurately determined in this experiment, again did not differ between Triton-treated animals and untreated controls in either lean or obese rats, and the values were all low (Table III). Thus, as was true for the chow-fed animals, little newly synthesized FA could have been transferred from liver to adipose tissue in either group of animals during the period of this experiment.

Total carcass FA were measured as 5 ± 0.7 and 25 ± 1.7% of body weight, respectively, in these lean and obese rats. By the arguments already used for the chow-fed animals, 10.5 g of average total body adipose tissue in the lean group should accumulate ca. 19 μmol newly made FA/hr, again substantially less than the value in extrahepatic carcass, which averaged ca. 60 μmol/hr (Table II). For the obese group, on the other hand, their 72 g average adipose tissue would accumulate 182 μmol newly

synthesized FA/hr, again sufficient to account for the observed average accumulation of 175 μmol/hr in the carcass of this group (Table II).

DISCUSSION

The relative amounts reported here for newly synthesized FA found in liver and adipose tissues at the end of a 1-hr pulse with tritiated water are similar to those given by Godbole and York (5); what is at issue is whether this pattern also represents the relative rates of synthesis in these tissues, or whether it is the result of large-scale redistribution between these tissues during the period of the experiment. Godbole and York calculated the total amounts of newly made FA which accumulated in the liver and (estimated) total adipose tissues in 5-week-old obese rats to be about equal, ca. 200 μmol/hr each. However, since hepatectomy reduced the accumulation in the adipose tissues by 90%, they concluded that the true rates of FA synthesis in intact rats must have been 380 μmol/hr in liver vs ca. 20 μmol/hr in adipose tissues, and consequently, that about half of the labeled FA newly synthesized in the liver had been transferred to adipose tissue within the 1-hr period of the experiment.

TABLE II

Accumulation of Newly Synthesized Fatty Acids in Tissues of High-Glucose, Fed-Refed Lean and Obese Zucker Rats 1 Hr Following Injection of ^3H_2O[a]

Animals	Triton[b]	Newly synthesized fatty acids				Calculated average adipose tissue
		Liver	Carcass	Adipose tissue SC	Adipose tissue PM	
		μmol/1 hr		μmol/g·hr		μmol/1 hr
Lean	-	26 ± 6	67 ± 4	1.6 ± 0.8	2.6 ± 0.3	19
		ns[d]	ns	ns	ns	
	+	30 ± 8	51 ± 7	1.0 ± 0.3	2.0 ± 0.4	
Obese	-	133 ± 8	205 ± 53	2.5 ± 0.3	3.1 ± 0.6	182
		ns	ns	ns	ns	
	+	96 ± 42	156 ± 32	2.1 ± 0.8	2.4 ± 0.7	

[a]Mean ± SE from 4 rats/group. Average body weights: lean 174 g, obese 239 g.

[b]Triton WR-1339 (200 mg/ml, 600 mg/kg BW) was injected iv into unanesthesized rats.

[c]See Table I, footnote d.

[d]ns: not significant.

TABLE III

Percent Distribution of Newly Synthesized Fatty Acids in Tissues of Zucker Rats after a 1-Hr Pulse with ^3H_2O in vivo[a]

Dietary state	Triton	Newly synthesized FA found in:			Calculated average adipose tissue
		Liver	Plasma	Carcass	
(I) Fed ad libitum					
Lean	-	8.2 ± 1.8	<1[b]	91.8 ± 1.8	6
	+	14.8 ± 1.1	<1	85.4 ± 1.0	
Obese	-	20.6 ± 4.9	<1	79.4 ± 5.0	78
	+	22.8 ± 1.5	<1	77.3 ± 1.5	
(II) High-glucose fed/refed					
Lean	-	27.0 ± 5.1	1.7 ± 0.3	71.4 ± 5.4	21
	+	37.0 ± 3.4	2.2 ± 0.4	60.8 ± 3.0	
Obese	-	35.7 ± 3.1	1.3 ± 0.2	63.3 ± 2.3	62
	+	35.0 ± 6.4	1.9 ± 0.4	63.1 ± 6.4	

[a]Mean ± SE from 4 rats/group. Conditions are described in Tables I and II.

[b]There was no detectable radioactivity in plasma aliquots used to determine activity.

[c]See legend to Table I, footnote d.

The validity of these conclusions obviously depends critically on the hepatectomy procedure used to establish this drastic redistribution. A priori, its magnitude seems startling, considering the short span of the experiment. Since transfer from liver to adipose tissue occurs via secretion and uptake of VLDL-TG, the maximal rate of transfer can not exceed the total flux of TGFA through that compartment. We may estimate this rate for the obese rats used by Godbole and York (5) to be between 30 and 60 mg TG/hr. The higher value was calculated from their study as follows: we assumed their animals had moderately hyperlipemic plasma corresponding to a plasma TG concentration of 150 mg/dl (4), a plasma vol of 4.5% of body weight and a total plasma TG pool size of 7.5 mg. Accepting the 0.1-hr half-life of Intralipid found by these authors as valid for VLDL-TG, the maximal flux through this plasma compartment in their study would have been 7.5 × 0.69/0.1 = 50 mg TG/hr. A lower estimate (30 mg TG/hr) is based on our own observed rate of TG secretion, using Triton WR-1339, corrected for difference inthe plasma vol of our 6-week-old and their 5-week-old obese rats. Since secretion of 50 mg TG/hr equals 200 μmol FA/hr, which also was the amount of newly made FA postulated to have been transferred from liver to adipose tissue during the 1-hr experiment, this interpretation would require both (a) that the liver secreted only such FA as had also been synthesized de novo during that time, and (b) that all TGFA secreted into plasma must be taken up and stored by adipose tissue. Both of these proposals are implausible. First of all, FA newly synthesized in liver mix with, and are substantially diluted by, FA derived from serum FFA and the continuous turnover of intracellular lipids in the liver. Second, the arrangement of the VLDL secretory apparatus in liver produces a considerable delay in the appearance of newly synthesized FA in serum TG within the time frame of 1 hr, as shown in Figure 2 of Godbole and York. Third, adipose tissue is by no means the only destination for plasma TGFA in the rat; a large proportion goes to other tissues, including substantial re-uptake into the liver itself (13; Kannan, et al., to be published). Thus, although the impact of these complicating circumstances on the results reported by Godbole and York cannot be quantitatively predicted with any certainty, these considerations a priori render it extremely unlikely that the massive transfer of newly synthesized FA from liver to adipose tissue proposed by Godbole and York could occur within their 1-hr experimental period. We therefore designed our experiments to demonstrate this point directly, by using Triton WR-1339 to block the uptake into adipose tissue of any newly synthesized FA secreted into plasma as VLDL-TGFA. As already discussed in Results, this blockage, indeed, had no significant effect on the amounts of newly made FA found in adipose tissue in any of our experimental groups, a result which immediately rules out any transfer on the scale proposed by Godbole and York. Furthermore, direct analysis of the TG which did accumulate in the plasma of Triton-treated animals showed them to contain very little newly synthesized FA, ca. 2% of the amounts calculated to be present in total adipose tissue in the high-glucose, fed-refed animals (Table III). Thus, only insignificant amounts of newly synthesized FA could have been transferred from the liver to adipose tissue in the controls not treated with Triton WR-1339. This, in fact, is also the conclusion to be drawn from the serum data reported by Godbole and York (5). These authors point to their finding of larger amounts of radioactive serum TGFA in obese compared to lean animals as further support for their postulated massive transfer of newly synthesized FA from liver to adipose tissue in the obese rats; however, a simple quantitative analysis of their data proves that, in their experiments, also only a small fraction of the radioactivity found in adipose tissue FA could have been transported there from the liver via plasma. The total amount of radioactivity lost from the plasma TGFA compartment in 1 hr (q_p [1 hr]) can be calculated from the relationship:

$$q_p \text{ (1 hr)} = A \text{ (1 hr)} L_{pp},$$

where A (1 hr) = the area (dpm.min) under the serum ^{3}H-TGFA vs time curve for 0-1 hr, and L_{pp} = the fractional rate constant of plasma TGFA turnover (min^{-1}). Using 0.11 min^{-1} for the L_{pp}, corresponding to the 6 min half-life given by Godbole and York, and the measured area under the curve in their Figure 2 (135 × 10^3 dpm.min), we obtain q_p (1 hr) = 135 × 10^3 dpm.min × 0.11 min^{-1} = 15,000 dpm. From this value, we may calculate (11) that 1.7 μmol of newly synthesized, liver-derived FA was lost from the plasma compartment of these obese rats in 1 hr. Thus, even if exclusively taken up by adipose tissue, this again would represent only ca. 1% of the 200 μmol/ newly synthesized FA calculated by Godbole and York to be present in total perimetrial and subcutaneous adipose tissues of their obese animals. Clearly then, both in our experiments and in those of Godbole and York, the contribution of the liver to the newly synthesized FA

found in adipose tissue at 1 hr was entirely negligible. Thus, the interpretation of our own findings and those of Godbole and York is exactly opposite of theirs: the amounts of newly synthesized FA found in adipose tissue at 1 hr are straightforward measurements of the FA synthesis in situ in that tissue. Once this has been established, there remains little difference in interpretation of our experiments and those of Godbole and York (5), other than an accounting for the gross artifact produced by surgical hepatectomy. Here, the depression of lipogenesis in the adipose tissue of obese animals found by Godbole and York most likely reflected the depression of plasma glucose and insulin also found in these animals. Although the authors point to the fact that a similar effect also was seen in lean, hepatectomized animals, which did not show significantly depressed adipose tissue lipogenesis, this could result simply from the fact that the rates in lean animals were an order of magnitude lower than those in the obese; thus, severely decreased glucose uptake into the adipose tissue of hepatectomized animals could well be rate-limiting for lipogenesis only in the obese rats.

It should, of course, be stressed that this data in no way shows that transport of the newly synthesized FA from the liver is an insignificant source of adipose tissue depot FA; it merely demonstrates that its kinetics make this transfer undetectable in short-term experiments of the kind discussed here. We have stressed the importance of distinguishing between the transport of tracer and tracee in our other studies of TGFA transport from liver to adipose tissue in both mice and rats (Kannan, Elovson, Lyon, Mead and Baker, manuscripts in preparation). The results of these kinetic studies, and simple considerations of mass balance in the steady state, suggests that a considerable portion of newly synthesized FA deposited in the adipose tissue of both lean and obese rats are made in the liver, especially in those nutritional conditions where the liver accounts for one-third to one-half of total body FA synthesis.

ACKNOWLEDGMENT

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Influence of Lecithin on the Chromatography of Steroidal Glucosiduronates in Chloroform/Formamide

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ABSTRACT

The interaction between lecithin and steroidal glucosiduronates was investigated by use of partition chromatography in chloroform/formamide and infrared spectroscopy. It was observed that lecithin increases the solubility of both glucosiduronic acids and esters in chloroform and concluded that this phenomenon occurs because of the formation of hydrogen bonds between the phosphodiester group of lecithin and hydroxyl groups of the steroid conjugates.

INTRODUCTION

Phosphatidylcholine (lecithin) is a major component of animal cell membranes and it plays a significant role in the transport of various substances in body fluids and across membranes. Thus, the interaction of lecithin and other naturally occurring compounds is of considerable importance. Lecithin is known to increase the solubility of several compounds in relatively nonpolar solvents. Kendall (1) observed that epinephrine, which normally is insoluble in chloroform, is soluble in this solvent in the presence of lecithin. In studies on carbohydrates, Taylor and Taylor (2) found that lecithin promotes the solubilization of glucose, glucuronolactone and related compounds in benzene. Burstein (3) reported that androstenalone sulfate, which is relatively polar, was extractable from water in the presence of lecithin, and Furasawa et al. (4) found that the solubility of various carboxylic acids in chloroform is considerably increased in the presence of lecithin.

Our prior interest in the use of alkylammonium compounds in chromatography systems (5,6) to increase the solubility of steroidal glucosiduronates in relatively nonpolar organic solvents led us to consider how lecithin, which contains a phosphodiester group and a quarternary ammonium group, would interact with glucosiduronic acids and glucosiduronic esters. This paper describes the influence of lecithin on the chromatographic mobility of glucosiduronic acids and esters in $CHCl_3$-$HCONH_2$ and the influence of lecithin and tetraheptylammonium chloride on the infrared (IR) spectra of the glucosiduronic esters in chloroform.

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EXPERIMENTAL

Steroidal glucosiduronates were synthesized and characterized in our laboratory. Chloroform and formamide used in chromatography were of reagent grade. Chloroform for IR spectroscopy was freed of alcohol by passing it through a column of alumina (neutral, activity grade I) and stored over sodium hydroxide pellets in the dark. Sources of other chemicals were as follows: tetraheptylammonium chloride (TA•Cl), Eastman Organic Chemicals, Rochester, NY; tri-*n*-octylamine (TOA), K&K Laboratories, Inc., Plainview, NY; lecithin for chromatography (vegetable, purified), Mann Research Labs., New York, NY; lecithin for IR experiments (synthetic β-γ-dipalmitoyl-D-L-α-glycerlphosphoryl choline), Sigma Chemical Co., St. Louis, MO.

Chromatographic procedures have been described previously (5,7). Chromatography was carried out in a sealed jar that contained chloroform and was lined with paper that was impregnated with formamide. A sheet of paper was drawn through a mixture (3:7, v/v) of stationary phase (0.0-0.8 M salt in formamide) and methanol; about 15 min later, the solutes were applied. The chromatogram was developed by allowing equilibrated mobile phase (0.05-0.10 M additive in chloroform) to flow down the paper. Steroidal glucosiduronates were located by viewing the papers over 254 nm radiation or by treating them with 0.4% tetrazolium blue/3 N NaOH (1:9). Formamide was removed (6) from the papers prior to treatment with the alkaline tetrazolium blue reagent. The front of the solubilizer (lecithin, TOA, or TA•Cl) that was present in the mobile phase on the chromatogram was visible after treatment of the paper with the alkaline tetrazolium blue reagent.

Infrared spectra were taken on a Beckman IR-18 instrument using matched NaCl cells which have a path length of 0.10 mm.

RESULTS AND DISCUSSION

Eight steroidal glucosiduronic acids and the corresponding methyl esters were chromatographed on paper in chloroform/formamide with and without various additives in the mobile phase. Results are summarized as R_F values in Table I.

None of the glucosiduronic acids migrated measurably from the origin in the parent system of chloroform/formamide. When the chloroform phase was ca. 0.05 M with lecithin, all of the acids migrated significantly and their migration increased still further when the concentration of lecithin was increased to 0.10 M. Parallel runs in which the mobile phase was 0.10 M with lecithin, tri-*n*-octylamine (TOA) (5) and tetraheptylammonium chloride (TA·Cl) (5) indicated that lecithin has about the same effect in increasing the mobility of the glucosiduronic acids as does the free amine TOA but much less effect than the quaternary ammonium halide TA·Cl.

The R_F values of the methyl esters also increased when lecithin was added to the mobile phase and the change in R_F was found to parallel the change in concentration of the lecithin. Addition of TOA to the mobile phase did not increase the migration of the esters and in a few instances, it retarded their movement slightly. In general, the change in R_F of the esters when lecithin was added to the system was similar in magnitude to that found when an equivalent molar amount of TA·Cl was used.

The effect of the various additives on the partition of the steroidal glucosiduronates between chloroform and formamide can be evaluated quantitatively by converting R_F values to R_M values; $R_M = \log(\frac{1}{R_F} - 1)$. The change in R_M for a specific compound when

TABLE I

R_F Values for Steroidal Glucosiduronates in Chloroform/Formamide with Different Solubilizers in the Mobile Phase

Compound[a]	Additive[b] in mobile phase			
	None	Lecithin[c]	TOA[d]	TA·Cl[e]
EGA	<0.01	0.016[f]	0.027	0.024	0.79
SGA	<0.01	0.027[f]	0.043	0.044	0.86
BGA	<0.01	0.018[f]	0.027	0.035	0.78
AGA	<0.01	0.018[f]	0.030	0.043	0.79
QGA	<0.01	0.038[f]	0.057	0.14	0.90
THS 3-GA	<0.01	0.011[f]	0.018	–	–
THA 3-GA	<0.01	0.010[f]	0.014	0.024	0.50
THQ 3-GA	<0.01	0.016[f]	0.029	0.088	–
EGMe	0.010	0.13[f]	0.25	0.010	0.16
SGMe	0.029	0.26[f]	0.36	0.025	0.27
BGMe	0.025	0.26[f]	0.38	0.025	0.26
AGMe	0.077	0.30[f]	0.41	0.060	0.37
QGMe	0.28	0.63[f]	0.66	0.22	0.62
THS 3-GMe	0.019	0.20[f]	0.28	–	–
THA 3-GMe	0.044	0.16[f]	0.25	0.034	0.25
THQ 3-GMe	0.23	0.42[f]	0.48	0.15	–

R_F values for the acids were based on the front of the additive; R_F values for the esters were based on the chloroform front.

[a]Glucosiduronic acids are abbreviated GA, corresponding methyl esters are abbreviated GMe. EGA = 17α-hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; SGA = 17α-hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; BGA = 11β-hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; AGA = 3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; QGA = 3,20-dioxopregn-4-en-21-yl β-D-glucopyranosiduronic acid; THS 3-GA = 17α,21-dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid; THA 3-GA = 21-hydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid; THQ 3-GA = 21-hydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid.

[b]Mobile phase was ca. 0.10 M with additive.

[c]Lecithin was vegetable in origin, probably dimyristoyl.

[d]Tri-*n*-octylamine.

[e]Tetraheptylammonium chloride.

[f]Mobile phase was ca. 0.05 M with lecithin.

TABLE II

R_F Values for Steroidal Glucosiduronates with 0.10 M Lecithin in the Mobile Phase of Chloroform/Formamide and with Various Salts in the Stationary Phase

Compound[a]	Additive[b] in stationary phase				
	None	KCl		KI	LiCl
EGA	0.027	0.049[c]	0.085	0.063	0.15
SGA	0.043	0.074[c]	0.14	0.11	0.22
BGA	0.027	0.041[c]	0.074	0.058	0.12
AGA	0.030	0.041[c]	0.072	0.056	0.12
QGA	0.057	0.095[c]	0.17	0.12	0.27
THS 3-GA	0.018	0.024[c]	0.028	0.028	0.048
THA 3-GA	0.014	0.020[c]	0.020	0.017	0.029
THQ 3-GA	0.029	0.039[c]	0.059	0.048	0.10
EGMe	0.25	–	0.31	0.28	0.31
SGMe	0.36	–	0.45	0.44	0.51
BGMe	0.38	–	0.48	0.43	0.52
AGMe	0.41	–	0.50	0.43	0.52
QGMe	0.66	–	0.75	0.76	0.79
THS 3-GMe	0.28	–	0.38	0.37	0.40
THA 3-GMe	0.24	–	0.33	0.28	0.35
THQ 3-GMe	0.48	–	0.62	0.59	0.63

R_F values for the acids were based on the lecithin front; R_F values for the esters were based on the chloroform front.

[a]See footnote to Table I for explanation of abbreviations.

[b]Stationary phase was 0.80 M with salt.

[c]Stationary phase was 0.20 M with salt.

chromatographed in the presence and in the absence of a substance added to increase solubility can be interpreted as approximately equal to the log of the change in partition coefficient (conc in the mobile phase/conc in the stationary phase). When the mobile phase of chloroform/formamide was made 0.10 M with lecithin, the partition coefficient of the glucosiduronic acids was increased 2- to 6-fold. When TA·Cl was used under comparable conditions, the partition coefficient of the acids was increased 100- to 200-fold. These values are minimal amounts; although all R_F values of the acids in the solvent system without additives are <0.01, the calculations are based on the assumption that each R_F value was 0.01.

In contrast to the foregoing observations with the acids, lecithin is even more effective in increasing mobility of the esters than is TA·Cl. Addition of lecithin to the mobile phase of the chromatography system to give a 0.10 M solution causes a 3.3- to 32-fold increase in the partition coefficient of the esters whereas the largest increase in partition coefficient caused by an equivalent amount of TA·Cl was 19-fold.

Incorporation of KI, KCl or LiCl into the chromatography system led to a significant increase in the mobility of both the acids and esters (Table II) and the order of increasing effect on mobility was KI < KCl < LiCl. The fact that incorporation of these salts into the system which contained lecithin increased mobility of the acids rather than retarding it indicates that the effect is not produced by an ion-exchange mechanism (5). This conclusion is supported by the finding that KCl did not pass into $CHCl_3$ and react with the ionic centers of lecithin when 0.2 M lecithin-$CHCl_3$ was equilibrated with 0.5 M KCl/formamide; under such conditions, the concentration of KCl in the $CHCl_3$ phase was only 0.0007 M greater than in an analogous partition experiment in which no lecithin was included in the $CHCl_3$. In view of the foregoing observations, it is presumed that salts exert their effect on the partition by "salting out" the glucosiduronates from the formamide phase.

Previously, it has been reported (8) that the phosphodiester group of lecithin forms a hydrogen bond with the hydroxyl group of cholesterol and we have confirmed this observation. We found that with 0.05 M cholesterol in chloroform the hydroxyl band at 3610 cm^{-1} was shifted to 3350 cm^{-1} ($\Delta\bar{\upsilon}$ = 260 cm^{-1}) in the presence of 0.20 M lecithin whereas it was shifted to 3360 cm^{-1} ($\Delta\bar{\upsilon}$ = 250 cm^{-1}) by 0.20 M TA·Cl; this observation implies that lecithin forms stronger H-bonds with cholesterol under these conditions than does TA·Cl. As indicated by values for the equilibrium constants, various phosphate esters form particularly strong H-bonds (9) with hydroxyl groups of diverse

compounds and formation of these bonds is accompanied by large decreases in the frequency of the hydroxyl band.

We have shown also that lecithin forms hydrogen bonds with THQ 3-GMe (Fig. 1), a typical steroidal glucosiduronic ester. In the

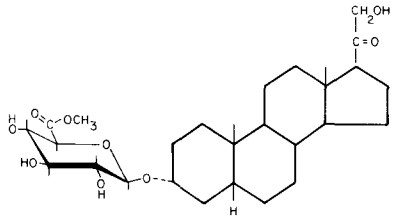

FIG. 1. Structure of THQ 3-GMe.

presence of lecithin, the intensity of the band for the unbonded hydroxyl vibration (Fig. 2a, 3600 cm^{-1}, dotted line) is less than its intensity in the presence of TA•Cl (Fig. 2b, 3600 cm^{-1}, dotted line). In addition, the shift of the band is greater in the presence of lecithin (3600 to 3220 cm^{-1}) than of TA•Cl (3600 to 3280 cm^{-1}).

In the carbonyl region, the IR spectrum of THQ 3-GMe contains 2 bands (Fig. 3); the carbonyl group of the ester function absorbs at 1748 cm^{-1} and the G-20 carbonyl group at 1707 cm^{-1}. Addition of TA•Cl or lecithin alters the absorption of the carbonyl functions. The absorbance at 1748 cm^{-1} increases upon addition of TA•Cl (Fig. 3b) and this increase is accompanied by a decrease in absorbance at ~1730 cm^{-1}. We postulate that electron-rich Cl of TA•Cl competes with the ester carbonyl in forming an H-bond with the C-4′ hydroxyl group. As a result, the absorbance of the H-bonded carbonyl, which occurs at about 1730 cm^{-1} and is masked by the more intense bands at 1748 and 1707 cm^{-1}, is diminished and the absorbance of the unassociated carbonyl group is increased. A similar phenomenon probably occurs in the presence of lecithin (Fig. 3a) but the carbonyl groups of lecithin absorb so much of the incident radiation at 1750-1720 cm^{-1} that the instrument does not respond properly.

The absorbance of the C-20 carbonyl group at 1707 cm^{-1} decreases upon addition of either TA•Cl or lecithin. We postulate that this decrease results from formation of an H-bond between the additives and the C-21 hydroxyl group. Whether the change in spectrum is associated with the breaking of an intramolecular H-bond between the C-21 hydroxyl group and the C-20 carbonyl group or to some other mechanism is unclear since there is controversy concerning whether intramolecular hydrogen bonding actually occurs with steroids which have this type of structure (10-13).

Frequently, an investigator who is attempting to isolate a natural product from biological sources will extract an aqueous suspension or solution with a nonpolar solvent in order to remove unwanted material. The marked influence which lecithin may have on increasing the solubility of various classes of polar compounds in nonpolar solvents implies that an investigator should be wary of the possible presence of lecithin in extracts of natural products and the effect it may have on the partition of polar compounds between polar and nonpolar solvents. Although it has been

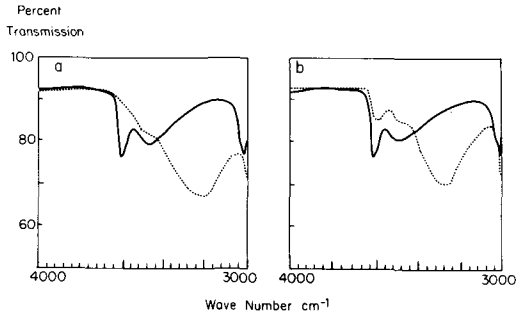

FIG. 2. Influence of lecithin (a) and TA•Cl (b) on the absorbance of the hydroxyl groups in THQ 3-GMe in $CHCl_3$. The solid line represents the IR spectrum of 0.05 M THQ 3-GMe in $CHCl_3$ with $CHCl_3$ in the reference cell; the dotted line represents the IR spectrum of 0.05 M THQ 3-GMe in 0.20 M additive-$CHCl_3$ with 0.20 M additive-$CHCl_3$ in the reference cell.

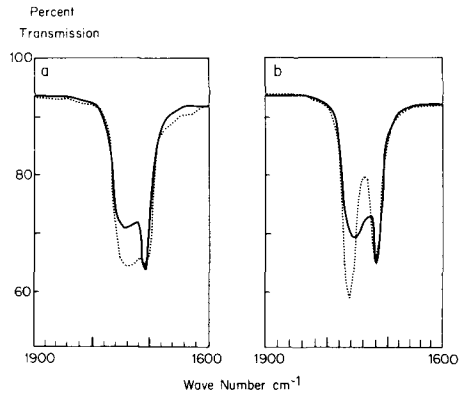

FIG. 3. Influence of lecithin (a) and TA•Cl (b) on the absorbance of the carbonyl groups in THQ 3-GMe in $CHCl_3$. The solid line represents the IR spectrum of 0.05 M THQ 3-GMe in $CHCl_3$ with $CHCl_3$ in the reference cell; the dotted line represents the IR spectrum of 0.05 M THQ 3-GMe in 0.20 M additive-$CHCl_3$ with 0.20 M additive-$CHCl_3$ in the reference cell.

demonstrated that lecithin can increase the solubility of polar compounds in the less polar phase during extraction in a separatory funnel and during chromatography on sheets of paper, we postulate that it can exert the same influence in appropriate solvent systems during chromatography in columns and on thin layers.

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Sterols of the Oyster, *Crassostrea virginica*

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ABSTRACT

A commercial sample of the oyster, *Crassostrea virginica*, obtained from Maryland waters of the Chesapeake Bay, contained 31 desmethylesterols and at least eight 4-monomethylsterols. The combined gas liquid chromatography-mass spectra of the minor components showed the presence of 6 unusual sterols, 24-ethylcholest-22-en-3β ol, 4α-methyl-24-ethylcholestan-3β-ol, occelasterol, (24E)-24-propylidene-cholest-5-en-3β-ol, (24Z0-24-propylidene-cholest-5-en-3β-ol, and 24-methylene-cholestanol. The C-24 configuration of 24-ethylcholest-5-enol, 24-methyl-cholesta-5,22-dienol, and 24-ethyl-cholesta-5,22 dienol were elucidated by 220 MHz nuclear magnetic resonance spectrometry.

INTRODUCTION

Many investigations have pointed out that the bivalves are unique in containing a great diversity of Δ^5-sterols. However, the origin of sterols in the bivalves is not fully elucidated. Rubinstein and Goad (1) reported that the marine diatom, *Phaeodactylum tricornutum*, contained epibrassicasterol, (24S)-24-methyl-cholesta-5,22E-dienol, as a principal sterol, and suggested that diatoms probably are the primary source of this sterol which is one of the prominent sterols in the bivalves and some marine invertebrates (2-4). Kobayashi and Mitsuhashi (5) also have suggested that epibrassicasterol is far more abundant in marine environments than brassicasterol, (24R)-24-methyl-cholesta-5,22E-dienol. Thus, the C-24 configuration of C_{28}- and C_{29}-sterols has received great interest in relation to the origin of sterols in marine invertebrates. Except for a few studies, however, the C-24 configuration has not been sufficiently characterized in the sterols of marine invertebrates, especially with the stanols and monoene sterols with saturated side chains of C_9 and C_{10}. This primarily results from the difficulty in separating them by sophisticated chromatographic techniques.

Lipophilic Sephadex column chromatography has been used for the separation of scallop sterols containing a complex mixture of C_{26}-C_{30} sterols, and then assigned the C-24 configuration of C_{28}- and C_{29}-sterols by high resolution (220 MHz) nuclear magnetic spectrometry (NMR) (6,7). The sterols of the oyster, *Crassostrea virginica*, have been investigated by several workers (8,9). However, the characterization of minor components is not fully accomplished. Therefore, we have reexamined the sterols of the oyster, *C. virginica*, and paid careful attention to the presence of methylsterols and other minor desmethylsterols which will provide significant information on the biosynthesis and modification of exogenous sterols in the oyster and other bivalves.

MATERIALS AND METHODS

Chromatography

Gas liquid chromatography (GLC) was performed on a Glowall Chromalab A-110 equipped with an argon ionization detector and a glass column (1.8 m x 3.4 mm id) of 3.0% SE-30 at 250 C (10). Relative retention times (RRT) were relative to cholesteryl acetate unless specified. Thin layer chromatography (TLC) was carried out using the following adsorbents and solvent systems: adsorbent—Silica Gel G, 10% (w/w) $AgNO_3$-Silica Gel G, and 20% (w/w) $AgNO_3$-Silica Gel $HF_{254+366}$; solvents—chloroform (commercial reagent grade) (system I), chloroform/methanol (98:2) (system II), chloroform/methanol (65:35) (system III), ethanol/free chloroform (system IV), and hexane/benzene (5:2) (system V). Sterols were detected under ultraviolet (UV) light after spraying Rhodamine 6G or by heating after spraying 10% H_2SO_4 in ethanol. Sterols or steryl acetates on the TLC plates were eluted with ether. Column chromatography of lipids on Silica Gel 60 (150 g) eluted with 150 ml each of 0 and 2% methanol in chloroform and 750 ml each of 6, 10, 15, 25 and 50% methanol in chloroform was carried out to separate steryl esters, free sterols and steryl sulfates; the eluates were checked by TLC on Silica Gel G with system III. Column chromatography of unsaponifiable matter on alumina (Woelm, grade II-III) was performed by increasing the proportions of ether in hexane (11). Column chromatography on 20% (w/w) $AgNO_3$-impregnated silicic acid (Bio-Sil® A,

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100-220 mesh, Bio-Rad Laboratories, Richmond, CA) ($AgNO_3$-column chromatography) was conducted by increasing the proportions of benzene in hexane (12). Column chromatography on a lipophilic Sephadex, the hydroxyalkoxypropyl derivative (13), was carried out according to the Patterson et al. method (6).

Physicochemical and General Methods

Mass spectra were obtained on a LKB 9000 mass spectrometer equipped with a Varian SS100 Data System. For combined GLC and mass spectrometry (GLC-MS), the LKB 9000 mass spectrometer was coupled with a column of 0.75% SE-30 (1.8 m x 4 mm id) and operated under the following conditions: GLC column temperature 230 C, electron energy 70 eV, accelerating voltage 3.5 kV, ion source temperature 290 C and electron multiplier 3.7 kV. NMR spectra were measured on a Varian HR-200 spectrometer (220 MHz, sweep width 250 Hz in $CDDl_3$. Acetylation of sterols was carried out with dry pyridine/acetic anhydride (1:1, v/v) at room temperature. Hydrolysis of steryl acetates was done with 5% KOH in ethanol at 70 C for 1 hr. Steryl sulfates were solvolyzed to free sterols by refluxing with 2% acetic acid in dioxane at 80 C for 24 hr.

Isolation of Sterols

The oysters, *C. virginica*, 12.0 kg in fresh weight, were obtained from a commercial source, September 21, 1979. Lipids (117.5 g) were extracted with chloroform/methanol/water by the Bligh and Dyer method (14). Aliquots of lipids (14.0 g) were separated into steryl ester, free sterols and steryl sulfate fractions by column chromatography on Silica Gel 60. Steryl esters, free sterols and steryl sulfates were mainly eluted with 0, 2 and 10-15% methanol in chloroform, respectively. The steryl ester and free sterol fractions were separately saponified, and the unsaponifiable matters were chromatographed on alumina (50 g) with 200 ml each of 0, 10, 20, 30, 40, 50 and 70% ether in hexane and 100% ether. Sterols were eluted with 30-40% ether in hexane. The steryl sulfate fraction was solvolyzed, and the free sterols obtained were purified by alumina column chromatography and TLC on Silica Gel G with system I. The percentage composition of sterols from the 3 sterol fractions was determined by GLC.

The remainder of lipids (103.5 g) was saponified, and the unsaponifiable matters were chromatographed on alumina (1 kg) with 2,000 ml each of 0, 10, 20, 30, 40 and 70% benzene in hexane and 100% benzene. In this alumina column chromatography, methylsterols and desmethylsterols were mainly eluted with 10% and 20-50% ether in hexane, respectively. The desmethylsterols (6.5 g) were acetylated and the acetate derivatives purified by alumina column chromatography. Recrystallization from methanol afforded desmethylsterol acetates (7.5 g). The methylsterol fraction obtained by the initial alumina column chromatography was contaminated with a small amount of desmethylsterols. Rechromatography of the crude methylsterol fraction on alumina afforded the mixture of methylsterols free of desmethylsterols. The isolated methylsterols (296 mg) gave a single spot in TLC on Silica Gel G with system II, but they were shown to be heterogenous by GLC. The methylsterols were less polar than common desmethylsterols but more polar than dimethylsterols such as lanosterol, so they were suspected to be a mixture of 4α-methylsterols.

Fractionation and Characterization of Desmethylsterols

The desmethylsterol acetates (7.5 g) were chromatographed on 20% (w/w) $AgNO_3$-silicic acid (440 g) with 1,500 ml each of 0, 10, 15, 20, 27, 29, 33, 36, 40, 50, 60 and 75% benzene in hexane and 100% benzene. Seventy-one fractions (each fraction, 300 ml or 250 ml) were collected and monitored by GLC and/or GLC-MS.

Fraction 15. Fractions 1-14 gave no steryl acetate. Fraction 15, eluted with 15% benzene in hexane, afforded a mixture of Δ^0- and Δ^5-steryl acetates. The 10% $AgNO_3$-TLC of fraction 15 with system V gave 3 bands. The less polar band was composed of cholestan-3β-yl (4a), 24-methylcholestan-3β-yl (7a), 24-ethylcholestan-3β-yl (10a) and a C_{30}-steryl (RRT 1.89) acetate (10d). The C_{30}-steryl acetate was deduced to be a 4α-monomethylsteryl acetate (see Results). GLC-MS: 4a (RRT 1.03), m/e 430 (M^+, 42%), 415 (M^+-CH_3, 7%), 370 (M^+-AcOH, 49%), 355 (M^+-AcOH-CH_3, 35%), 316 (M^+-C-1 to C-4, 9%), 290 (M^+-R-27, R = side chain, 13%), 276 (M^+-R-41, 50%), 275 (M^+-R-42, 35%), 257 (M^+-R-AcOH, 14%), 230 (M^+-R-27-AcOH, 41%), and 215 (M^+-R-42-AcOH, 100%); 7a (RRT 1.34), m/e 444 (M^+, 39%), 429 (M^+-Ch_3, 6%), 384 (M^+-AcOH, 32%), 330 (M^+-C-1 to C-4, 6%), 290 (7%), 276 (7%), 275 (36%), 257 (9%), 230 (36%), and 215 (100%); 10a (RRT 1.67), m/e 458 (M^+, 36%), 443 (M^+-CH_3, 4%), 398 (M^+-AcOH, 27%), 383 (M^+-AcOH-CH_3, 23%), 344 (M^+-C-1 to C-4, 5%), 290 (16%), 276 (29%), 257 (13%), 230 (40%), and 215 (100%). The middle band

contained small amounts of a C_{29}-steryl acetate (9a) (RRT 1.41) (see Results). The polar band was composed of a mixture of Δ^5-steryl acetates.

Fractions 16-19. These fractions, eluted with 20% benzene in hexane, afforded a mixture of cholest-5-en-3β-yl (4b), 24-methylcholest-5-en-3β-yl (7b) and 24-ethylcholest-5-en-3β-yl (10b) acetates. The mass spectra and RRT of these compounds corresponded with those reported for the authentic materials (10,15). The free sterols from the mixture of 4b, 7b, and 10b were chromatographed on lipophilic Sephadex for separation into individual components. 24-Ethylcholest-5-enol was isolated as a pure compound (a single peak in GLC), purified by recrystallization from methanol, and analyzed by NMR (220 MHz) to determine the C-24 configuration. Insufficient 24-methylcholest-5-enol was available for NMR analysis.

Fraction 20. Fraction 20, eluted with 20% benzene in hexane, afforded the mixture of 4b and 24-ethylcholesta-5,22-dien-3β-yl acetate (9b). The RRT and the GLC-MS of 9b corresponded to that of authentic 24-ethylcholesta-5,22-dien-3β-yl acetate (10,15). 9b was separated from 4b by 10% $AgNO_3$-TLC with system IV, and the C-24 configuration of 9b was evaluated by NMR spectrometry (see Results).

Fractions 21 and 22. These fractions, eluted with 25% benzene in hexane, gave 24-methylcholesta-5,22-dienyl acetate (6b) and a small amount of 4 compounds, 1b (RRT 0.64), 3b (RRT 0.91), 9b and 2b. The RRT and the GLC-MS were those of 24-methylcholesta-5,22-dienyl acetate (10,15). 6b was purified by 10% $AgNO_3$-TLC with system V and the C-24 configuration was elucidated by NMR spectrometry (see Results). The extremely minor component 2b was deduced to be occelasteryl acetate (see Results).

Fractions 23-26. These fractions, eluted with 25% benzene in hexane, afforded cholesta-5,22E-dien-3β-yl (3b) and 24-norcholesta-5,22-dien-3β-yl (1b) acetates, both of which were identified by GLC and GLC-MS.

Fractions 27-31. These fractions, eluted with 27% benzene in hexane, contained large amounts of 1b and (24E)-24-ethylidenecholest-5-en-3β-yl acetate (11b) and small amounts of desmosterol acetate (5b), plus 2 unknown compounds (RRT, 1.54 and 1.88). One of the unknown compounds (RRT 1.88) was characterized as (24E)-24-propylidenecholest-5-en-3β-yl acetate (13b), by GLC-MS (see Results). GLC-MS: 11b (RRT 1.63), m/e 394 (M^+-AcOH, 71%), 379 (M^+-AcOH-CH_3, 13%), 296 (M^+-C-23 to C-29 in 1H-AcOH, 100%), 281 (m/e 296-CH_3, 53%), 267 (7%), 255 (14%), 253 (29%), 228 (29%), and 213 (41%).

Fractions 32-34. These fractions, eluted with 27-29% benzene in hexane, yielded a large amount of (24Z)-24-ethylidenecholest-5-en-3β-yl acetate (12b) and small amounts of 5b and 2 unknown compounds (RRT 0.92, 1.93). GLC-MS: 5b (RRT 1.09), m/e 366 (M^+-AcOH, 80%), 351 (M^+-AcOH-CH_3, 23%), 342 (M^+-C-22 to C-27-1H, 1%), 255 (13%), 253 (32%), 228 (7%), 296 (M^+-C-23 to C-29-1H-AcOH, 100%), 281 (58%), 267 (5%), 255 (5%), 253 (13%), 228 (12%), and 213 (20%). One of the unknown compounds (RRT 1.93) was characterized as (24Z)-24-propylidene-cholest-5-en-3β-yl acetate (14b) by GLC-MS (see Results). Another unknown compound (RRT 0.92) was assumed to be a C_{27}-diene steryl acetate with the chemical structure closely related to 22-dehydrocholesteryl acetate.

Fractions 35-41. These fractions, eluted with 29-33% benzene in hexane, gave 2 major components (RRT 1.18 and 1.30) and 2 very minor components (RRT 0.92 and 1.05). One of them (RRT 1.30) was identified as 24-methylenecholestan-3β-yl acetate (8a) (see Results). Other compounds were not characterized in this study.

Fractions 42-44. These fractions afforded 24-methylenecholest-5-en-3β-yl acetate (8b) as a sole component.

Fractions 45-71. These fractions, eluted with 45-75% benzene in hexane, afforded 7 compounds (RRT 0.69, 0.95, 1.09, 1.20, 1.43, 1.55 and 1.76) which were deduced to be $\Delta^{5,7}$-steryl acetates and an unknown compound (RRT 1.69). The characterization of these compounds will be the subject of a later report.

RESULTS AND DISCUSSION

Sterol Composition

This study showed that the oyster, *C. virginica,* contains steryl esters besides the large amounts of free sterols (Tables I and II). Also, careful examination demonstrated the presence of steryl sulfates as a very minor constituent in the oyster. Some difference in the percentage composition of sterol components was observed among the 3 sterol fractions. The percentages of 22-dehydrocholesterol and cholesterol were higher in steryl esters than in free and sulfated sterols, whereas those of C_{29}-sterols, e.g., 24-ethylcholesta-5,22-dienol, 24-ethylcholest-5-enol and 24-ethylidenecholest-5-enol, were higher in the latter 2 sterol fractions than in steryl esters. The proportions of C_{28}-sterols did

TABLE I

Lipid and Sterol Content in the Oyster

Fraction	Composition (%)	
Lipids (% of fresh oysters)	0.98	
Unsaponifiable matters (% of lipid)	22.90	
Total sterols (% of lipid)	6.57	
Desmethylsterols	6.29	(95.7%)[a]
Methylsterols	0.28	(4.3%)
Esterified sterols	0.98	(14.9%)
Free sterols	5.58	(84.9%)
Sulfated sterols	0.01	(0.2%)

[a]Numbers in parentheses indicate the percentage of total sterols.

not differ markedly among the 3 sterol fractions.

The steryl sulfate is known to play an important role as a precursor of steroid hormone (16) and as an excretory material in mammals and insects (17,18). With respect to the marine invertebrates, the starfish, *Asterias rubens*, has been shown to contain steryl sulfates in a comparatively high concentration (19-21). Although the function of steryl sulfates in the oyster, *C. virginica*, is obscure, the occurrence of steryl sulfates will be of interest from the viewpoint of sterol metabolism in marine mollusks.

Alumina column chromatography of the unsaponifiable matters afforded a small amount of methylsterols, besides large amounts of desmethylsterols. The methylsterols were composed of one major component (RRT 1.14, 68% of total methylsterols) and 6 minor compounds. The characterization of these methylsterols is in progress at our laboratory.

The sterol components of desmethylsterols were characterized by GLC, GLC-MS, or NMR after the fractionation of $AgNO_3$-column chromatography. As a result, the desmethylsterols of the oyster, *C. virginica*, were shown to be a more complex mixture than reported in earlier studies (8,9). The desmethylsterols of the oyster are composed of Δ^5-sterols and small amounts of Δ^0- and $\Delta^{5,7}$-sterols. The prominent components of desmethylsterols were cholesterol (34.0%), 24-methylcholesta-5,22-dienol (15.6%), 24-methylenecholesterol (12.6%), 22-dehydrocholesterol (10.2%), 24-norcholesta-5,22-dienol (4.0%), 24-methylcholest-5-enol (2.6%), 24-ethylcholesta-5,22-dienol (2.0%), and the unidentified 7 $\Delta^{5,7}$-sterols (6.6%) (Table III). In addition to these sterols, 15 sterols were detected as a minor component (less than 1.0% of total desmethylsterols). Some of them were characterized by GLC-MS (Table IV).

Unconventional Sterols

Six unusual sterols were identified as very minor constituents. On $AgNO_3$-column chromatography, fraction 15 contained 2 unknown compounds, 9a (RRT 1.41) and 10d (RRT 1.89). The GLC-MS of 9a gave the molecular ion at m/e 456 corresponding to a C_{29}-monoene steryl acetate and other prominent ions at m/e 413 (M^+-43), 353 (M^+-43-AcOH), 344 (M^+-C-22 to C-29-1H), 329 (M^+-C-22 to C-29-1H-CH_3), 315 (M^+-R-2H), 284 (M^+-C-22 to C-29-1H-AcOH), 269 (M^+-C-22 to C-29-1H-AcOH-CH_3), 257 (M^+-R-AcOH), 255 (M^+-R-2H-AcOH), and 215 (M^+-R-42-AcOH) (Table IV). The intense ion at m/e 257 and the ion at m/e 215 established that 9a involved the side chain of $C_{10}H_{19}$ (MW = 139) containing one double bond. The presence of the high fragment ion at m/e 315, together with the ions at m/e 413, 353, 284 and 269, place the side chain double bond at Δ^{22} (15,22). On the basis of these data, this compound was identified as 24-ethylcholest-22-en-3β-yl acetate (9a). 24-Ethylcholest-22-en-5β-ol has been isolated from the slime mold, *Distyostelium discoideum*, by

TABLE II

Composition of the Sterols Isolated from the Esterified, Free and Sulfated Sterol Fractions (%)

GLC		Composition (%)			
Peak	RRT	Steryl ester	Free Sterol	Steryl sulfate	Main constituent
1	0.64	7.7	3.4	5.0	24-Norcholesta-5,22-dienol
2	0.91	17.1	9.1	8.4	22-Dehydrocholesterol
3	1.00	41.4	33.8	26.8	Cholesterol
4	1.12	12.6	16.1	11.5	24-Methylcholesta-5,22-dienol
5	1.29	11.8	14.0	10.2	24-Methylenecholesterol
6	1.41	0	8.2	9.3	24-Ethylcholesta-5,22-dienol
7	1.67	9.4	14.9	28.7	24-Ethylcholest-5-enol and 24-Ethylidenecholest-5-enol

Heftmann et al. (23). Erdman and Thomson (24) also have found monoene sterols with the unsaturated double bond in the side chain in the sponge, *Hymeniacidon perleve*. However, 24-ethylcholest-22-en-3β-ol has not been found in other marine environments.

Another compound (RRT 1.89) gave the molecular ion at m/e 472, corresponding to a C_{30} fully saturated stanyl acetate, and prominent ions at m/e 412 (M^+-AcOH), 397 (M^+-AcOH-CH_3), 290 (M^+-R-41), 271 (M^+-R-AcOH), 244 (M^+-R-27-AcOH), 230 (M^+-R-AcOH-41), and 229 (M^+-R-AcOH-42). The intense ions at m/e 229 (the highest above m/e 200) and 230, together with the molecular ion at m/e 412, established that this compound has the steroid ring with an extra methyl group and the side chain of $C_{10}H_{21}$ (MW = 141). Since

TABLE III

Sterol Components of Desmethylsterols from the Oyster

Sterol		RRT	%	Sterol		RRT	%
(1b)	24-Norcholesta-5,22-dienol	0.64	4.0	(8a)	24-Methylenecholestanol	1.30	0.5
	Unknown[a]	0.69	0.5	(7a)	24-Methylcholestanol	1.34	<0.1
(2b)	Occelasterol	0.87	<0.1	(9a)	24-Ethylcholest-22-enol	1.41	<0.1
(3b)	(*trans*)-22-Dehydrocholesterol	0.91	10.2	(9b)	24-Ethylcholesta-5,22-dienol	1.42	2.0
	Unknown	0.92	<0.1		Unknown[a]	1.43	0.2
	Unknown[a]	0.95	1.0		Unknown	1.54	<0.1
(4b)	Cholesterol	1.00	34.0		Unknown[a]	1.55	0.8
(4a)	Cholestanol	1.03	0.3	(10b)	24-Ethylcholest-5-enol	1.63	3.7
	Unknown	1.05	<0.1	(11b)	(24E)-24-Ethylidenecholesterol	1.63	1.5
(5b)	Desmosterol	1.09	0.2	(10a)	24-Ethylcholestanol	1.67	<0.1
	Unknown[a]	1.09	1.8	(12b)	(24Z)-24-Ethylidenecholesterol	1.69	4.6
(6b)	24-Methylcholesta-5,22-dienol	1.12	15.6		Unknown	1.69	0.2
	Unknown	1.18	0.4		Unknown[a]	1.76	0.2
	Unknown[a]	1.22	2.1	(13b)	(24E)-24-Propylidene-cholesterol	1.88	<0.1
(8b)	24-Methylenecholesterol	1.26	12.6	(14b)	(24Z)-24-Propylidenecholesterol	1.93	0.2
(7b)	24-Methylcholest-5-enol	1.30	3.7				

[a]$\Delta^{5,7}$-Sterols: 6.6% of total desmethylsterols.

TABLE IV

Mass Spectral Data for the Acetates of the Unusual Sterols in the Oyster

	Sterols					
Fragmentation	9a	10d	2b	13b	14b	8a
M^+	456(12)[a]	472(11)	–	–	–	442(8)
M^+-CH_3	–	–	–	–	–	427(16)
M^+-43	413(16)	–	–	–	–	–
M^+-43-AcOH	353(23)	–	–	–	–	–
M^+-AcOH	–	412(23)	366(47)	408(42)	408(42)	382(3)
M^+-AcOH-CH_3	–	379(18)	351(5)	393(7)	393(7)	367(13)
M^+-(cleavage at C-20, 22 + 1H): a	344(51)	–	–	–	–	–
M^+-a-CH_3	329(14)	–	–	–	–	–
M^+-a-AcOH	284(8)	–	282(11)	–	–	–
M^+-a-AcOH-CH_3	269(17)	–	267(3)	–	–	–
M^+-(cleavage at C-22,23 + 1H): b	–	–	–	–	–	358(100)
M^+-b-CH_3	–	–	–	–	–	343(29)
M^+-b-AcOH	–	–	–	296(100)	296(100)	298(21)
M^+-b-AcOH-CH_3	315(88)	–	–	281(58)	81(58)	283(23)
M^+-R-2H	–	–	–	–	–	315(76)
M^+-R-41	–	290(9)	–	–	–	–
M^+-R-42	–	–	–	–	–	275(25)
M^+-R-AcOH	257(100)	271(7)	255(34)	255(2)	255(12)	257(12)
M^+-R-2H-AcOH	255(21)	–	253(8)	253(8)	253(30)	255(35)
M^+-R-27-AcOH	–	244(9)	228(4)	228(11)	228(39)	230(25)
M^+-R-41-AcOH	–	230(34)	–	–	–	–
M^+-R-42-AcOH	215(22)	229(40)	213(12)	213(24)	213(45)	215(92)
Other ions	–	43(100)	43(100)	–	–	229(34)

[a]Relative intensity

the mass spectrum of this compound did not yield a high peak at m/e 441 (M^+-CH_3) characteristic of a sterol with a 14 α-methyl group (25), the extra methyl group in the steroid ring was conceived to be located at C-4α from biogenetic grounds. Ballantine et al. (26) and Steudler et al. (27) have found 24-propylcholestanol in an oceanic sponge, *Synops* sp., and 4-methylgorgostanol in the zooxanthellae of the gorgonian, *Briareum asbestinum*, respectively. However, the possibility of these 2 C_{30}-stanols was clearly denied by the mass spectral pattern. Therefore, this steryl acetate was identified as 4α-methyl-24-ethylcholestan-3β-yl acetate (10d) which also has been found in the scallop, *Patinopecten yessoensis* (28).

The very minor component (2b, RRT 0.87) was present in the fractions eluted with 25% benzene in hexane. The mass spectrum of this compound gave prominent ions at m/e 366 (M^+-AcOH), 351 (M^+-AcOH-CH_3), 282 (M^+-C-22 to C-28-1H-AcOH), 267 (M^+-C-22 to C-28-1H-AcOH-CH_3), 255, 253, 228, 213 and 43. The mass spectral cracking pattern of this compound was similar to that of 22-*trans*-22-dehydrocholesteryl acetate (3B). However, this compound gave a shorter RRT in GLC and eluted faster in $AgNO_3$-column chromatography than 3b. These data showed that this compound was occelasteryl acetate, 22-*trans*-24-nor-(24S)-methylcholesta-5,22-dien-3β-yl acetate, which was first isolated from the marine annelid, *Pseudopotamilla occelata* (5).

The fractions, eluted with 27-29% benzene in hexane, afford 2 C_{30} steryl acetates, 13B (RRT 1.88) and 14b (RRT 1.93). The mass spectra of 13b and 14b were similar and gave the prominent ions at m/e 408 (M^+-AcOH), 393 (M^+-AcOH-CH_3), 296 (M^+-C-23 to C-30-1H-AcOH), 281 (M^+C23 to C-30-1H-AcOH-CH_3), 255, 253, 228 and 213. The absence of molecular ion peak showed that both compounds were C_{30}-Δ^5-steryl acetates. The ions at m/e 255 and 253, together with the ion at m/e 408, revealed the presence of unsaturated side chain of $C_{11}H_{21}$ (15). The intense ions at m/e 296 and 281 (22) were indicative of the $\Delta^{24(28)}$ bond. Considering the RRT in GLC and the mobility in $AgNO_3$-column chromatography besides the MS data, 13b and 14b were identified as (24E)-24-propylidenecholest-5-en-3β-yl and (24Z)-24-propylidene-cholest-5-en-3β-yl acetates, respectively. Both compounds have been first found in the scallop, *Placopecten magellanicus* (29,30).

Fractions 35-41, eluted with 29-33% benzene in hexane, contained the unknown compound 8a with a RRT of 1.30 as a major component. The mass spectrum of 8a gave the molecular ion at m/e 442 corresponding to a C_{28}-monoene steryl acetate and the prominent ions at m/e 427 (M^+-CH_3), 382 (M^+-AcOH), 367 (M^+-AcOH-CH_3), 358 (M^+-C-23 to C-28-1H), 343 (M^+-C-23 to C-28-1H-CH_3) 298 (M^+-C-23 to C-28-1H-AcOH), 283 (M^+-C-23 to C-28-1H-AcOH-CH_3), 315 (M^+-R-2H), 275 (M^+-R-42), 257 (M^+-R-AcOH), 255, 230, 229 and 215. The ions at m/e 315, 257 and 215, together with the low intensity of the molecular ion at m/e 442, showed the presence of the ring-saturated steryl acetate with one double bond in the side chain. The ions at m/e 358, 343, 298 and 283 were conceived to be formed by a McLafferty rearrangement (22) and indicated the $\Delta^{24(28)}$ bond. From these data, this compound was identified as 24-methylenecholestanyl acetate (8a) which had been first isolated from the sponge, *Hymeniacidon perleve* (24).

C-24 Configuration of C_{28} and C_{29}-Sterols

The C-24 configuration of 3 steryl acetates from the oyster was evaluated by the 220 MHz NMR at a sweep width of 250 Hz (Table V). The NMR spectra of epibrassicasterol (24S/24α) and brassicasterol (24R/24β) have been shown to be similar, but a significant downfield shift of the C-21 methyl proton signal in comparison of the spectra of the 2 compounds has been observed in the brassicasterol (7,31). This difference is useful for the discrimination of the C-24 configuration of both sterols. In the acetates of the 2 sterols, a similar difference has been seen (1,31). As shown in Table V, the NMR spectrum of 24-methylcholesta-5,22-dienyl acetate from the oyster showed that the isolated compounds were a mixture of both the 24S and 24R epimers. Khalil et al. (7) also have pointed out the concurrence of brassicasterol and epibrassicasterol in the sterols of the scallop, *Placopecten magellanicus*.

The C-24 epimers of C_{29}-$\Delta^{5,22}$-sterols have been observed to be not easily differentiated as compared with those of C_{28}-$\Delta^{5,22}$-sterols (31-33). However, since the C-21 and C-29 signals are deshielded in the 24R/24β epimer (poriferasterol) more than in the 24S/24α epimer (stigmasterol), both isomers are differentiated by the discrepancy in the shapes of peaks in the δ 0.77-0.86 region (31,32). The NMR spectrum of 24-ethylcholesta-5,22-dienyl acetate from the oyster indicated that the isolated steryl acetate contained predominantly poriferasteryl acetate (Table V).

The C-24 epimers of C_{29}-Δ^5-sterols also have been realized to be differentiated by the

TABLE V

Methyl Group Chemical Shifts of C_{28}- and C_{29}-Sterols Isolated from the Oyster

Sterols	C-24 Config.	Chemical shift (δ) of methyl group						
		C-18	C-19	C-21	C-26	C-27	C-28	C-29
Sterols from the oyster								
24-Methylcholesta-5,22-dienol acetate	*	0.690	1.020	1.002	0.833	0.815	0.908	–
24-Ethylcholesta-5,22-dienol acetate	*2	0.693	1.020	1.026	0.844	0.791	–	0.808
24-Ethylcholest-5-enol	*2	0.674	1.005	0.920	0.831	0.806	–	0.846
Standards								
Brassicasterol acetate*3	R/β	0.693	1.023	1.003	0.832	0.815	0.909	–
Epibrassicasterol acetate*3	S/α	0.693	1.018	1.001	0.833	0.816	0.907	–
Stigmasterol acetate*3	S/α	0.693	1.017	1.017	0.842	0.791	–	0.799
Poriferasterol acetate*3	R/β	0.695	1.020	1.025	0.841	0.791	–	0.808
β-Sitosterol*3	R/α	0.680	1.007	0.919	0.833	0.813	–	0.841
Clionasterol*4	S/β	0.683	1.001	0.928	0.834	0.814	–	0.855

* Predominantly the S/α isomer; δ of C-21 methyl of R/β isomer = 1.007.
*2Predominantly the R/β isomer.
*3Cited from the data of Rubinstein et al. (31).
*4Cited from the data of Khalil et al. (7).

signals of C-29 methyl group; the C-29 methyl signal of the 24S/24β epimer (clionasterol) appeared in the deshielded region compared with that of the 24R/24α epimer (β-sitosterol). The NMR spectrum of 24-ethylcholest-5-enyl acetate from the oyster showed that the isolated steryl acetate was almost completely composed of the 24S/24β epimer, clionasteryl acetate (Table V).

Khalil et al. (7) have shown the concurrence of the 24α- and 24β-epimers in the C_{28}- and C_{29}-sterols isolated from the scallop, *P. magellanicus*. This study also revealed the presence of both 24α- and 24β-epimers in 24-methylcholesta-5,22-dienyl acetate from the oyster. However, in C_{29}-sterols, e.g., 24-ethylcholesta-5,22-dienyl acetate and 24-ethylcholest-5-enyl acetate, the oyster contained, almost exclusively, the 24β-epimer.

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Dietary Fibers: V. Binding of Bile Salts, Phospholipids and Cholesterol from Mixed Micelles by Bile Acid Sequestrants and Dietary Fibers

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ABSTRACT

Mixed micelles were prepared containing combinations of either taurocholate or taurochenodeoxycholate, monoolein, oleic acid, dioleylphosphatidylcholine (lecithin) and cholesterol. These were incubated with commercial bile-acid-sequestering resins, cholestyramine and DEAE-Sephadex, or various dietary fibers and fiber components including wheat bran, cellulose, alfalfa, lignin and 2 viscosity grades of guar gum. Binding was determined as the difference between the radioactivity of each micellar component added and that recovered in the centrifugal supernatant after incubation. In general, the extent of bile salt sequestration was characteristic and reproducible for each bile salt, and was largely unaffected by the presence of one or more additional components of the micellar mixture, including the other bile salt. Cholestyramine bound 81-92% of the bile salts and 86-99% of the phospholipid and cholesterol present in micelles. DEAE-Sephadex sequestered only 49% of the taurocholate and 84% of the taurochenodeoxycholate, but completely removed all of the phospholipid and cholesterol from micelles containing either bile salt. Among the dietary fibers, guar gum of either viscosity bound between 20-38% of each micellar component, whereas lignin, alfalfa, wheat bran and cellulose were progressively less effective in sequestration of individual components of mixed micelles. The extent of sequestration of micellar components by these resins and fibers is reasonably correlated with the effects of these same materials on lymphatic absorption of lipids and to their suggested hypocholesteremic properties.

INTRODUCTION

It has been well documented that certain natural dietary fibers and purified fiber constitutents have potential bile acid binding capacities when tested in vitro (1-7). These studies generally have been conducted using bile acid concentrations well above those required for formation of multimolecular detergent aggregates referred to as micelles (8). In general, the bile acid sequestering ability of various dietary fibers has been compared to those of commercial ion exchange resins (2,4,5,7) which are used therapeutically to increase bile excretion (9) and reduce hypercholesteremias (10). There also is suggestive evidence that dietary fiber components, e.g., pectin, may increase bile acid excretion in vivo (11), thereby altering bile acid balance, affecting cholesterol absorption (12) and reducing hypercholesteremia (13).

In our earlier studies on direct effects of dietary fibers on lymphatic absorption of cholesterol and triglyceride fatty acids (7,14), we also conducted ultrastructural studies on the small intestine and colon of rats fed cholestyramine and various dietary fibers (15-17). It was found that prolonged (6-week) ingestion of 2% levels of cholestyramine, colestipol or Sechol-ex®, and to a lesser extent 15% levels of alfalfa or pectin, resulted in accumulation of lipid in the small intestinal mucosa (M.M. Cassidy and G.V. Vahouny, unpublished observations), suggesting an interference with lipid transport from the cell. Interference of lymphatic absorption of cholesterol and triglyceride are directly correlated (7,14). The major requirements for intestinal cellular lipid transport are related to availability of surface "coat" or membrane components, namely specific apolipoproteins and phospholipids (18). Interference with availability of these components can result in cellular lipid accumulation and reduced lipid absorption (e.g., ref. 19).

Since one major source of the phospholipid component of intestinal lipoprotein membranes is ultimately derived from the lecithin of bile (20), it seemed possible that bile acid sequestering agents might also bind phospholipids present in mixed micelles with bile salts, and thereby interfere with reabsorption of lecithin and the subsequent availability for lipoprotein membrane synthesis.

The present studies were designed to test this premise using concentrations of bile salt, phospholipid, monoglyceride and fatty acid previously shown to form soluble mixed micelles (21), and in vitro conditions used earlier to test bile salt-sequestering abilities of various resins and dietary fibers (7).

MATERIALS AND METHODS

Monoolein, oleic acid and the sodium salts of taurocholic and taurochenodeoxycholic acids were obtained from Sigma Chemical Co., St. Louis, MO. Cholesterol was purchased from Supelco, Bellefonte, PA. The [7α-^{3}H] bile salts and [1-^{14}C] dioleylphosphatidylcholine were from New England Nuclear Corp., Boston, MA. [1,2-^{3}H] cholesterol and [4-^{14}C] cholesterol were purchased from Amersham-Searle, Arlington Heights, IL.

Mixed micelles containing monoolein and oleic acid and various combinations of the bile salts, phospholipid and cholesterol were prepared as previously described (21). This first involved determination of the critical micellar concentrations of each component when mixed in different combinations (21). The concentrations of each component were within physiological ranges and included 5 mM bile salt, 250 μM monoolein, 500 μM oleic acid, 625 μM phospholipid and 250 μM cholesterol. In addition, the mixture contained various combinations of the tritium- and carbon-labeled components for analysis by liquid scintillation spectrometry. Micellar medium was prepared by mixing the lipids in hexane with the bile salt in physiological saline. After evaporation of the hexane at 25 C under nitrogen, complete solution was achieved by mechanical agitation on a tube buzzer (Vortex) for 30-60 sec. The solutions were then centrifuged at 105,000 G for 1 hr and the clear infranatant, representing micellar bile salts and lipids, was analyzed by liquid scintillation spectrometry.

Binding of bile salts and lipids was determined as previously described by Hagerman et al. (22) and Kritchevsky and Story (4) and as employed earlier in this laboratory (7). The test materials included cholestyramine (Questran; from Dr. H.P. Sarett, Mead Johnson, Evansville, IN); DEAE-Sephadex (Secholex®, a gift from Dr. A Howard, Cambridge, England); cellulose (Solka FLOC, Brown and Co., Berlin, NH); alfalfa and white wheat bran (Bio-Serv, Frenchtown, NJ); lignin (Westvaco, Charleston, SC); and 2 viscosity grades of guar gum (very high viscosity, Hathway; low viscosity, food grade, Hercules, Inc., Wilmington, DE). Forty mg of the appropriate resin or fiber source was added to 5 ml of each micellar solution in a stoppered tube and the mixture was shaken in a Dubnoff incubator at 37 C for 1 hr. The tubes were centrifuged at 30,000 G for 10 min and the entire supernatant was unified by mechanical agitation on a Vortex mixer prior to assay. Aliquots (0.1 ml) of the supernatant were added to 10 ml liquid scintillant (Scintiverse, Yorktown Research, Elmhurst, PA), and radioactivity was determined in a Beckman LS 250 liquid scintillation spectrometer using external standardization. Controls of the appropriate micellar media without added binding substances were carried through the same procedure. Binding was determined as the difference between the radioactivity of each micellar component added and that recovered in the supernatant after incubation. All studies were carried out at least 3 times and all isotope analyses were carried out in duplicate. Figures represent the means ± standard error of the mean.

RESULTS AND DISCUSSION

The conditions for determining bile acid sequestration from micellar solutions were established using cholestyramine as the test material. Table I summarizes the results of bile acid and cholesterol binding from mixed micelles containing monoolein and oleic acid. In all cases, binding of taurocholate ranged from 75 to 79% even when taurocholate was present in a 3:1 molar mixture with taurochenodeoxycholate. The binding of taurochenodeoxycholate ranged from 91 to 92% and this was unchanged in the presence of a 3-fold molar excess of taurocholate. The extent of binding of each of these bile salts and the differential binding of taurocholate and taurochenodeoxycholate are essentially identical to that reported previously (5) for bile salt binding by cholestyramine in the absence of other micellar components (see also Table VI). Thus, at the levels of fatty acid and monoolein used in these studies, there was no major influence of these amphiphiles on cholestyramine sequestration of the bile salts. Cholesterol sequestration from micelles containing either bile salt was essentially complete, as had been reported previously (7).

As shown in Table II, the presence of a second amphipath, dioleylphosphatidylcholine, in the micellar mixture had little or no effect on the sequestration of either bile salt or of cholesterol from the micellar mixture. This was not unexpected since the bile salt concentrations used in the present studies are below the maximal binding ability of 40 mg cholestyramine (2). Despite this, the differential affinity of the resin for taurochenodeoxycholate and taurocholate is still apparent.

Of considerable interest in this study was the extensive loss of the available phospholipid from micelles containing either bile salt. The apparent "binding" of lecithin was inversely related to the extent of binding of the bile salt. It seemed possible that the specific binding of bile salts from micellar media might

result in dissociation of the micelle and result in insolubility of the remaining micellar components. To test this, mixtures of the resin and micelles were centrifuged and the supernatant was assayed for isotope along the length of the tube. Under these conditions, "unbound" phospholipid and cholesterol were found largely as a floating layer at the top of the tube. Thus, in all studies, the supernatants from 30,000 G centrifugation of the resins or fibers were unified by extensive Vortex mixing prior to isotope analysis. As shown in Tables III and IV, this procedure generally provided uniform "binding" data with small statistical variations.

The equilibration of cholestyramine-bound bile salt and phospholipid with the aqueous micellar media was tested by incubating cholestyramine with an excess of either labeled amphipath, reisolation of the resin and subsequent reincubation in fresh micellar media. When [^{3}H] taurocholate-bound cholestyramine was subsequently reincubated in micellar media containing unlabeled bile salt alone (Table V, group 1), or in media containing both bile salt and phospholipid (Table V, groups 2 and 4), 18.0-22.4% of the bound, labeled taurocholate was released into the media. Thus, 77.6-82.0% of the label remained associated with the resin, a figure comparable to the resin binding of this bile salt from micellar solutions (see Tables I and II). A comparable release of labeled lecithin from the resin occurred during incubations in lecithin-containing micellar media (Table V, group 3), When the resin containing prebound

TABLE I

Binding of Micellar Bile Salts and Cholesterol by Cholestyramine

Micellar preparation[a]	Binding (%)	
	Bile salt	Cholesterol
Taurocholate	76.6 ± 0.1[b]	–
Taurocholate + cholesterol	75.2 ± 1.1	96.2 ± 0.4
Taurochenodeoxycholate	91.4 ± 0.1	–
Taurochenodeoxycholate + cholesterol	91.3 ± 0.4	97.0 ± 0.4
Taurocholate + taurochenodeoxycholate[c]	79.4 ± 0.5 TC 92.0 ± 0.2 TCDC	–

[a]Micelles contained 250 μM monoolein, 500 μM oleic acid, 5 mM bile salt and 250 μM [4-^{14}C] cholesterol where appropriate and were prepared according to Thornton et al. (21). These were shaken with 40 mg cholestyramine for 1 hr at 37 C and centrifuged at 30,000 G for 10 min. Binding was determined as the difference between labeled substances in the micellar media before and after incubation with the resin.

[b]The figures represent the means from 6 or more studies ± SEM.

[c]Total concentration of bile salts was 5 mM included in a 3:1 molar ratio of taurocholate:taurochenodeoxycholate. Duplicate incubations were carried out containing either [7α-^{3}H] taurocholate (TC) with unlabeled taurochenodeoxycholate (TCDC) or labeled [7α-^{3}H] TCDC and unlabeled TC.

TABLE II

Binding of Micellar Bile Salts, Phospholipids, and Cholesterol by Cholestyramine

Micellar preparation[a]	Binding (%)		
	Bile salt	Phospholipid	Cholesterol
Taurocholate + lecithin (TC-L)	79.3 ± 0.7[b]	91.0 ± 0.7	–
TC-L + cholesterol	–	92.1 ± 1.0	92.4 ± 0.9
Taurochenodeoxycholate + lecithin (TCD-L)	92.1 ± 0.1	74.6 ± 1.1	–
TCD-L + cholesterol	–	87.7 ± 1.0	88.3 ± 1.1

[a]Micelles contained 250 μM monoolein, 500 μM oleic acid, 5 mM bile salts, 625 μM dioleylphosphatidylcholine and 250 μM cholesterol as indicated. In each case, 2 of the micellar components were labeled and binding was determined as described in Table I and the text.

[b]Figures represent means from 6 studies ± SEM.

TABLE III

Binding of Components of Taurochenodeoxycholate-Phospholipid Micelles by Bile Acid Sequestrants and Dietary Fibers

Test substance[a]	Binding (%)		
	Bile Salt	Phospholipid	Cholesterol
Cholestyramine	91.9 ± 0.6[d]	99.0 ± 0.2	86.2 ± 4.8
DEAE-Sephadex	84.2 ± 0.3	99.4 ± 0.2	99.6 ± 0.1
Guar gum[b]	31.1 ± 2.7	21.7 ± 4.6	23.4 ± 0.7
Guar gum[c]	37.6 ± 3.0	33.6 ± 1.6	27.2 ± 0.5
Lignin	38.7 ± 1.4	1.6 ± 1.5	14.5 ± 0.7
Alfalfa	14.4 ± 1.1	1.5 ± 1.3	83.6 ± 0.6
Wheat bran	12.1 ± 0.7	0 ± 0	9.5 ± 0.8
Cellulose	3.5 ± 1.0	1.3 ± 1.0	4.7 ± 1.7

[a]Micellar mixtures contained 5 mM taurochenodeoxycholate, 625 μM lecithin, 250 μM monoolein, 500 μM oleic acid and 250 μM cholesterol. Triplicate incubations were carried out with 40 mg of each test substance and contained various combinations of [7α-^{3}H]taurochenodeoxycholate, [1-^{14}C]dioleylphosphatidylcholine and either [1,2-^{3}H]cholesterol or [4-^{14}C]cholesterol.

[b]Guar gum: low viscosity, food grade.

[c]Guar gum: high viscosity.

[d]Figures represent means from 6-12 incubations ± SEM.

TABLE IV

Binding of Components of Taurocholate-Phospholipid Micelles to Bile Acid Sequestrants and Dietary Fibers

Test substance[a]	Binding (%)		
	Bile salt	Phospholipid	Cholesterol
Cholestyramine	81.6 ± 0.1[d]	98.9 ± 0.3	95.3 ± 1.2
DEAE-Sephadex	49.1 ± 1.0	99.2 ± 0.5	99.6 ± 0.1
Guar gum[b]	35.6 ± 1.7	21.5 ± 1.8	22.7 ± 3.3
Guar gum[c]	31.7 ± 1.2	27.3 ± 2.1	18.3 ± 4.6
Lignin	20.2 ± 5.7	8.8 ± 3.4	4.7 ± 5.5
Alfalfa	6.7 ± 0.5	3.6 ± 1.1	1.1 ± 1.0
Wheat bran	3.6 ± 1.1	6.3 ± 2.0	0 ± 0
Cellulose	1.4 ± 0.3	0.5 ± 0.5	7.5 ± 1.9

[a]Micellar mixture contained 5mM taurocholate, 625 μM lecithin, 250 μM monoolein, 500 μM oleic acid and 250 μM cholesterol. Triplicate incubations were carried out with 40 mg of each test substance and contained various combinations of [7α-^{3}H]taurocholate, [1-^{14}C]dioleylphosphatidylcholine and either [1,2-^{3}H]cholesterol or [4-^{14}C]cholesterol.

[b]Guar gum: low viscosity, food grade.

[c]Guar gum: high viscosity.

[d]Figures represent means from 6-12 incubations ± SEM.

taurocholate was incubated in a mixed micellar medium containing labeled lecithin, there was quantitative adsorption of the phospholipid to the taurocholate-cholestyramine complex. These data imply that despite saturation of the ionic binding ability of the resin with bile salt, phospholipid binding was not decreased; it was perhaps even increased. Thus, there apparently are separate "binding" sites on the resin for this amphipath, which may be either hydrophilic or may represent hydrophobic association with the resin core. It also is possible that the amphipathic properties of the bile salt are unaltered by binding to the resin and that the prebound micellar bile salts are still capable of associating with other amphipaths and amphiphiles.

Having established that cholestyramine was able to effectively sequester various components of a mixed medium, we conducted studies on the "binding" of these same micellar components by a second ion-exchange, DEAE-Sephadex (Secholex®) and a variety of dietary fibers (Tables III and IV). DEAE-Sephadex is less effective than cholestyramine in binding micellar taurochenodeoxycholate (Table III) or taurocholate (Table IV) and this difference is comparable to that reported earlier using simple bile salt micelles (2). Despite the difference, the sequestration of phospholipid and cholesterol from micelles containing either bile salt was

TABLE V

Release of Cholestyramine-bound Bile Salt and Phospholipid into Aqueous Micellar Media

Component bound to cholestyramine[a]	Reincubation media[b]	Released into media (%)
Taurocholate	Taurocholate, 5 mM	22.4 ± 0.3
Taurocholate	Taurocholate + lecithin, 625 μM	18.0 ± 2.7
Lecithin	Lecithin	18.0 ± 2.1
Taurocholate	Taurocholate + [^{14}C]lecithin	(99.7 ± 0.1% binding of lecithin from reincubation media)

[a]Taurocholate (100 μmol) or dioleylphosphatidylcholine (2.5 μmol) were incubated with 40 mg cholestyramine for one hr at 37 C under conditions yielding maximal binding (2). The resin was reisolated by centrifugation at 30,000 G for 10 min and washed once with 0.9% sodium chloride.

[b]The reincubation micellar media contained monoolein, oleic acid and either bile salt (5 mM) or phospholipid (625 μM) as indicated. Incubations of the labeled resin with micellar media were carried out for 1 hr at 37 C.

essentially quantitative. Two different preparations of guar gum gave comparable results with either taurochenodeoxycholate or taurocholate micelles. In both cases, this fiber "sequestered" 31-38% of the bile salt, 21-34% of the phospholipid and 18-27% of the cholesterol from micellar solutions. Whether this represents hydrophilic or hydrophobic binding, entrapment of liquid crystals, or simply reflects entrapment of intact micelles in a gel matrix cannot be determined. In any case, this dietary fiber source has been reported to increase fecal elimination of bile salts and to have a hypocholesteremic effect in vivo (23).

As has been reported earlier (5), lignin, which is a hydrophobic component of dietary fibers has bile acid sequestering properties and apparently shows a greater affinity for taurochenodeoxycholate than for taurocholate. However, the sequestration of phospholipid and cholesterol from mixed micelles was relatively poor, implying that this fiber source does not associate with intact micelles. Results on the hypocholesteremic properties of lignin and on its ability to increase fecal excretion of bile acids have been inconsistent (24), probably because of differences in commercial sources of lignin and the variability in binding bile salts and other micellar components.

Alfalfa has been reported to have hypocholesteremic properties in experimental animals (25) and to reduce lymphatic absorption of cholesterol in rats (7). These effects have been attributed to a combination of bile acid sequestration by the dietary fiber component(s) of alfalfa (5) and to the interaction of the saponins of alfalfa with cholesterol (26). In the present studies, the binding of taurochenodeoxycholate to alfalfa was twice that observed with taurocholate and in both cases, sequestration of phospholipid was negligible. With taurochenodeoxycholate containing micelles, extensive sequestration of cholesterol by alfalfa was demonstrable, and this is compatible with our earlier findings (7) and with the effects of this dietary fiber on cholesterol absorption in rats (7). However, with taurocholate-containing micelles, cholesterol binding by alfalfa was negligible, implying a difference in availability of the sterol to the fiber. When mixed micelles containing both bile salts were employed (data not shown), micellar cholesterol was effectively sequestered by the fiber. The differential "binding" of cholesterol in the absence of significant phospholipid sequestration suggests that this is not an entrapment phenomena, and that sedimenting liquid crystals are not formed during bile salt sequestration by this fiber source.

Wheat bran and cellulose previously have been reported to have poor bile acid sequestering ability (2,5), and, unless used in high concentrations where intraluminal bulk phase diffusion is limiting, there is little direct effect on circulating cholesterol levels (see ref. 27). In the present studies, these fiber sources were generally ineffective in sequestering any of the measured components of mixed micellar solutions.

A comparison of the bile salt sequestering ability of cholestyramine and dietary fibers when the bile salts were presented alone as simple micelles (5), or were presented as more physiological mixed micelles is summarized in Table VI. It is apparent that other components of highly soluble, mixed micelles do not significantly influence the extent of bile acid binding by either the ion exchange resin or the dietary fibers tested.

The results of the present study might allow more reasonable predictions regarding the effi-

TABLE VI

Adsorption of Taurocholate and Taurochenodeoxycholate from Simple and Mixed Micelles

Test substance[a]	Bile salt			
	Taurocholate micelles		Taurochenodeoxycholate micelles	
	Simple[b]	Mixed	Simple[2]	Mixed
Cholestyramine	80.7	81.6	95.4	91.9
Lignin	22.1	20.2	25.4	38.7
Alfalfa	6.9	6.7	15.1	14.4
Wheat bran	1.4	3.6	9.8	21.1
Cellulose	1.0	1.4	0	3.5

[a]Conditions for bile salt binding were as described in Tables IV and V.
[b]Data taken from Story and Kritchevsky (5).

cacy of various fiber preparations in altering bile acid balance, increasing fecal bile acid excretion and reducing cholesterol absorption. High viscosity fibers such as guar gum and pectin are largely effective by altering bulk phase diffusion of intraluminal nutrients (28). In the water-soluble nutrients such as glucose, this diffusion apparently does not affect the total absorption of the nutrient (G.V. Vahouny and T. Roy, unpublished observations) (28), but apparently alters the rate of absorption and results in glucose transport over a larger portion of the small intestine (G.V. Vahouny and T. Roy, unpublished observations) (29). This reduces postalimentary hyperglycemia, but only when the fiber is provided with the diet (30). With respect to cholesterol absorption and bile acid balance, the high viscosity fibers can reduce intestinal cholesterol transport by "sequestering" micellar components, thereby reducing the availability of the amphipaths for solubilization of lipid digestion products, and ultimately resulting in increased bile acid excretion (1) and perhaps fecal excretion of other lipid materials such as phospholipids and cholesterol.

Because of the limited association of bile salts with lignin, and the apparent lack of affinity of other micellar components for this fiber, the effects of this fiber on bile acid excretion and on hypercholesteremias might be expected to be variable, depending on diet and lignin concentrations (27). Similarly, effects of nonbinding fibers such as wheat bran and cellulose on bile acid excretion and serum cholesterol levels have been reported to be negligible in most studies (see ref. 27). However, when concentrations of these fibers are sufficiently elevated to effectively interfere with bulk phase diffusion of nutrients in the intestinal lumen, they may interfere with cholesterol and fatty acid absorption (7), increase bile excretion (31) and result in limited reductions of serum cholesterol levels (see ref. 27).

Finally, the "bile acid sequestering" resins used in the present study show an apparent affinity in vitro for several components of mixed micelles. Whether this represents sequestration of intact micelles or binding of specific micellar components cannot be ascertained from this study. However, a similar affinity for the aggregates in vivo would be expected to reduce availability not only of bile salt for reabsorption in the lower lumen, but to result in decreased absorption of both sterol and triglyceride resulting, in part, from reduced availability of phospholipid for intestinal lipoprotein transport. This might explain, at least in part, the comparable effects of these materials on both cholesterol and triglyceride absorption (7,14), and the morphological evidence showing accumulation of lipid in the intestinal epithelial cells of animals given 2% levels of these resins for several weeks (M.M. Cassidy and G.V. Vahouny, unpublished observations). Comparable data in human subjects given Questran® and certain dietary fibers to reduce serum cholesterol levels have yet to be obtained.

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Cholesteryl Ester Hydrolase Activity in Human Symptomatic Atherosclerosis

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ABSTRACT

Acid cholesteryl ester hydrolase (CEH) activity was assayed in mononuclear cells of patients with symptomatic atherosclerosis (transient ischemic attacks, TIA) and in age-matched controls showing no evidence of atherosclerosis. The acid CEH level of TIA patients was significantly lower than that of controls (1074 ± 128 vs 2113 ± 255 pmol/mg P/hr, mean ± SE). Neither mononuclear cell nor plasma cholesterol and cholesteryl ester concentrations differed significantly between atherosclerotic and control groups. TIA women had lower mononuclear cell concentrations of free cholesterol than men.

INTRODUCTION

Atherosclerotic lesions are characterized by an increase in extracellular and intracellular lipids, particularly cholesteryl esters. Arterial accumulation of cholesteryl esters could result from a variety of metabolic imbalances: (a) an increased uptake of low density lipoprotein (LDL)-associated cholesterol into cells, (b) augmented cholesterol synthesis or cholesterol esterification within the smooth muscle cell, (c) diminished intracellular hydrolysis of esters, and (d) reduced "efflux" of cholesterol and its esters via high density lipoprotein (HDL) (1-5).

Although each of these metabolic alterations may play a part in cholesteryl ester deposition, we have focused our investigations on lysosomal cholesteryl ester hydrolase (CEH) in atherosclerosis. deDuve (6) proposed that a relative deficiency of this enzyme results in intracellular accumulation of cholesteryl esters in arterial smooth muscle cells. Two human diseases, cholesteryl ester storage disease (CESD) and Wolman's disease, are characterized by a deficiency of lysosomal cholesteryl esterase in all tissues studied (7,8). In CESD, extensive premature atherosclerotic changes accompany the enzyme deficiency; victims of Wolman's disease die in infancy of adrenal and liver failure before vascular changes occur.

In this study we investigate the possibility that altered acid CEH levels are not limited to these rare lysosomal storage diseases, but also occur in atherosclerosis. We measured the acid CEH in mononuclear blood cells of patients with symptomatic atherosclerosis, primarily those undergoing endarterectomy for transient ischemic attacks (TIA). The enzyme levels of these patients were compared to those of age-matched controls with no symptoms of atherosclerosis.

MATERIALS AND METHODS

Subjects

Volunteers were selected from patients admitted to the Health Sciences Center Hospital (Portland, OR) to form an approximately homogeneous group with respect to height, weight, sex and age. Control subjects, hospitalized for elective surgery, were free of symptomatic atherosclerosis. Patients with hyperlipidemia or abnormal laboratory tests of renal and hepatic function were excluded as controls. The atherosclerotic group consisted of individuals hospitalized for TIA of the brain. These patients, undergoing carotid endarterectomy, were free from diabetes. The mean age of the control group was 61.0 ± 2.6 years (range 43-72 years, female n=5, male n=6); that of the TIA group was 63.5 ± 2.8 years (range 46-77 years, female, n=6, male n=6).

Collection of Blood Samples

Samples of venous blood (20 ml in EDTA) were taken from each nonfasting subject in a uniform manner. All samples were obtained between 8 a.m. and 9:30 a.m. The samples were assayed immediately for mononuclear CEH activity and aliquots of plasma and mononuclear cells were processed for cholesterol and cholesteryl ester determinations.

Biochemical Analysis

The acid CEH activity of the mononuclear cells was measured as previously reported but will be described briefly (9,10). Optimal assay conditions for mononuclear cell CEH were determined for pH (4.5) and substrate concentrations (3-4 nmol). Hydrolysis was linear with incubation time (up to 90 min) and with protein concentration (40-120 μg/incubation).

Each 10 ml of blood was diluted with 30 ml normal saline and carefully layered upon 10 ml Lymphoprep (sodium metrizoate/Ficoll). The samples were then centrifuged for 30 min at 20 C, at 400 x g (measured at the interface between blood and Lymphoprep). The middle band, containing the mononuclear cells, was removed and centrifuged at 1000 x g to pellet the cells. The mononuclear cells were then washed several times with saline. After the final wash, the cell suspension was transferred to a 2.5-ml Dounce homogenizer. The cell suspensions were uniformly homogenized in saline 30 times prior to adding them to the CEH assay system. Disruption of the cells by more vigorous means failed to show any increased CEH activity in this assay system. Routinely, 1-1.5 x 10^6 cells were added to the incubation medium. The assay medium contained 1.3 ml 0.2 M acetate buffer, pH 4.5, 0.1-0.2 ml mononuclear cell homogenate, and 3-4 nmol [^{14}C]-1-cholesteryl oleate (SA 55 mCi/mmol, New England Nuclear, Boston, MA) injected in 20 μl acetone. In control samples, *p*-chloromercuriphenyl sulfonic acid (PCMPS) was added to inhibit the enzyme. At 3.3 x 10^{-3} M concentration, the PCMPS completely inhibited the CEH activity. After the incubation of the samples for 1 hr at 37 C, the samples were extracted with 3.0 ml benzene/chloroform/methanol, 1:0.5:1.2., 0.6 ml of 0.3 M NaOH was added, and the samples were stirred and centrifuged. A 0.5-ml aliquot of the upper phase (containing free oleic acid) was added to a toluene-10% Beckman Biosolv scintillation cocktail and the radioactivity determined. In this system, intra-assay variability is ±7%.

Plasma and mononuclear cell cholesterol and ester levels were measured in each subject. A 0.1-ml aliquot of plasma and a 0.25-ml aliquot of the mononuclear cell homogenate was extracted twice with 5 ml chloroform/methanol, 2:1. The samples were then evaporated to dryness, derivatized with TMS-Sil Prep (Applied Science Laboratories, State College, PA) and assayed on a 5830-A Hewlett-Packard gas chromatograph (3% SE-30 on 10% Gas Chrom, Applied Science Laboratories) to obtain free cholesterol levels. The samples were then saponified, extracted and again derivatized to obtain the total cholesterol concentration. The samples were corrected for procedural losses by including [^{14}C]cholesterol and [^{14}C]4-cholesteryl oleate standards in each assay. The recoveries for each compound were greater than 90% (range: 90-100%). The values for cholesterol and its ester are 10-15% lower than standard clinical colorimetric methods. Protein concentration in each mononuclear cells sample was determined by the Lowry et al. method (11). The statistical significance of mean differences was determined using Student's t-test.

RESULTS

The CEH measured in mononuclear cells from both control subjects and TIA patients exhibited maximal activity at pH 4.5 by the acetone dispersion assay. This acid optimum is characteristic of lysosomal hydrolases, in general, and corresponds to the pH optimum reported by other investigators for CEH activity associated with the lysosome fraction of liver, aorta and fibroblasts (12-14).

The CEH activities of control and endarterectomy (TIA) groups are shown in Table I. In controls, the mean CEH activity was 2113 ± 255, compared to 1074 ± 128 pmol/mg protein/hr in TIA patients (mean ± SE). This 2-fold difference in activity is highly significant ($P < 0.005$). While control men tend to have higher CEH activity than women, the difference is not significant. However, in control women and men, CEH levels were significantly higher than in their TIA counterparts. Plasma and mononuclear cell cholesterol concentrations for control and TIA groups are listed in Table I. Neither the total plasma cholesterol nor the mononuclear-cell free and esterified cholesterol concentrations differed significantly between the 2 groups.

The Pearson r test also was performed to reveal correlations between CEH activity and cholesterol concentration. No significant relationship between enzymatic activity and plasma or mononuclear-cell cholesterol was detected.

Control and TIA groups were composed of almost equal numbers of men and women, and we compared CEH activity, cholesterol and cholesteryl ester concentrations in women vs men. The only significant variation between women and men was a mononuclear-cell free cholesterol in the TIA group: in women, 5.16 ± 0.87 μg/mg protein; men 16.39 ± 3.09 μg/mg protein ($P < 0.005$).

DISCUSSION

In this study, we found significantly lower mononuclear-cell CEH activity in individuals with symptomatic atherosclerosis. In this respect, our findings are similar to those in Wolman's and CESD diseases. Several investigators have drawn a comparison between these lysosomal storage diseases, in which atherosclerosis appears in infancy of early childhood, and atherosclerosis which manifests itself in

TABLE I

Mononuclear Cell CEH, Cholesterol and Cholesteryl Ester in Atherosclerotic (TIA) and Control Subjects

	Control			TIA			P
Mononuclear CEH (pmol/mg protein/hr)							
Total	2113	± 255	(11)[a]	1074	± 128	(12)[a]	<0.005
Female	1841	± 267	(5)	982	± 115	(6)	<0.01
Male	2340	± 224	(6)	1166	± 223	(6)	<0.005
Mononuclear free cholesterol (μg/mg protein)							
Total	13.20	± 3.03	(11)	11.41	± 2.56	(10)	NS
Female	8.47	± 1.69	(5)	5.16	± 0.87	(5)	NS
Male	17.15	± 4.81	(6)	16.39	± 3.09	(5)[b]	NS
Mononuclear cholesteryl ester (μg/mg protein)							
Total	5.01	± 0.76	(11)	3.60	± 0.63	(10)	NS
Female	4.09	± 0.42	(5)	3.53	± 0.86	(5)	NS
Male	5.79	± 1.58	(6)	3.66	± 0.72	(5)	NS
Plasma total cholesterol (mg/dl)							
Total	183	± 14	(9)	207	± 9	(8)	NS
Female	182	± 32	(4)	232	± 10	(4)	NS
Male	183	± 25	(5)	193	± 35	(4)	NS

[a]=n.

[b]Mononuclear free cholesterol values for males vs females differ significantly in this group ($P < 0.005$).

middle or late life (3,15,16). In this gradually developing form of atherosclerosis, a relative, even *slight*, acid CEH deficiency may contribute to the accumulation of cholesteryl esters in arterial musculature. Alternatively, diminished CEH activity could be a secondary effect of elevated intracellular cholesterol concentration, rather than an inborn enzyme deficiency (6). Thus, excess amounts of intralysosomal cholesteryl esters may block the active sites of CEH, reducing its activity. Inasmuch as we found no correlation between enzymatic activity and mononuclear cell or plasma cholesteryl ester concentration, our data does not support deDuve's hypothesis nor distinguish between these 2 theories. However, since mononuclear blood cells are short-lived and many are mitotic, they are less likely to show cholesteryl ester accumulation than are the arterial smooth muscle cells. In addition, they have free access to HDL, which would facilitate removal of cholesteryl esters.

A sex difference was observed in mononuclear-cell free cholesterol in the TIA group; the reduced level in women probably is not directly related to an alteration in CEH activity, although hormones are known to modulate lipid metabolism at many levels (17-19). However, we have found that women taking oral contraceptives, known to have an increased incidence of early atherosclerosis (20,21), display changes in cholesterol and its esters in plasma, and in mononuclear cells, with reductions in their mononuclear-cell CEH activity (10).

To discover whether reduced mononuclear CEH activity is present *before* the onset of symptomatic atherosclerosis, will need further study. However, the demonstration of profoundly depressed CEH levels in Wolman's and cholesteryl ester storage diseases (7,8), the CESD with demonstrated atherosclerosis, and our own data that individuals with symptomatic atherosclerosis have significantly lower mononuclear-cell CEH activity, strengthen the theory which postulates a role of depressed CEH levels in atherosclerosis.

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Placental Transport of *trans* Fatty Acids in the Rat

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ABSTRACT

Placental transport of 9-*trans* [1-^{14}C] octadecenoic (elaidic) and 9-*trans*,12-*trans* [1-^{14}C] octadecadienoic (linoelaidic) acids was demonstrated in rats. On the 18th day of gestation, a ^{14}C-labeled albumin complex of elaidic or linoelaidic acid was injected into the jugular vein of pregnant rats. For comparison, 9-*cis* [1-^{14}C] octadecenoic (oleic) or 9-*cis*,12-*cis* [1-^{14}C] octadecadienoic (linoleic) acid also was injected into the maternal circulation of rats. All animals were sacrificed 1 hr following injection. Lipid composition and distribution of label were determined in maternal plasma, placental and fetal tissues. Differences in specific activities of plasma, placental and fetal total lipids indicated a decreasing concentration gradient for both *cis* and *trans* isomers of octadecenoic and octadecadienoic acids. Distribution of radioactivity in various lipid components was determined by thin layer chromatography. Irrespective of the label, the highest percentage of total radioactivity was carried by triglycerides (TG) in maternal plasma (∿60-80%), and was incorporated mainly in phospholipids (PL) of fetal tissues (∿50-60%). A nearly equal distribution of the label was found between PL and TG of placental lipids (∿40%). Radioactivity of fatty acid methyl esters (FAME) determined by radio-gas liquid chromatography indicated that after injection of linoelaidate, radioactivity of maternal plasma, placental and fetal tissue FAME was associated only with *t,t*-18:2. Following injection of elaidate, all the radioactivity in placental FAME was associated with *t*-18:1; however, in fetal tissues, the label was distributed between 16:0 and *t*-18:1. These findings suggest that, in contrast to linoelaidic acid, rat fetal tissues can metabolize elaidic acid via β oxidation to form acetyl CoA and palmitic acid.

INTRODUCTION

A constant supply of precursors from the mother is necessary for the growth and development of the fetus. Although glucose and certain glycolytic products may be the major precursors of fetal lipids and the major source of fetal energy, some preformed lipids are probably needed (1). From the earliest stages of development, fetal tissues can incorporate maternal fatty acids into complex lipids such as cholesteryl esters (CE), triglycerides (TG) and phospholipids (PL) (2). Fetal tissues also have the capacity to synthesize fatty acids, primarily producing palmitic acid (3). In addition, fetal tissues have the potential for β-oxidation of fatty acids (4,5) suggesting that fetal tissues may metabolize *trans* isomers of fatty acids.

Although transfer of saturated and *cis* unsaturated fatty acids has been demonstrated in rats, very few studies have investigated placental transport of *trans* fatty acids. In a number of studies, Johnston and coworkers reported little transfer of *trans* fatty acids across the human and rat placenta (6,7). Other investigators, however, have reported significant transfer of *trans* fatty acids across the rat placenta (8-10). In contrast to the findings of Johnston et al. (6), McConnell and Sinclair demonstrated deposition of elaidic acid in the bodies of newborn rats from mothers fed a diet rich in elaidic acid (8). Ono and Fredrickson reported placental transport of radioactively labeled *trans* isomers of oleic and linoleic acids (10). In addition, the occurrence of elaidic acid in newborn mice was recently reported (11). Although studies may suggest little placental transfer of *trans* fatty acids, experiments have not excluded the transfer and subsequent rapid oxidation of *trans* fatty acids by fetal tissues. Thus, the passage of dietary *trans* fatty acids into fetal tissues remains controversial.

This study was undertaken to investigate maternal-fetal transport of *cis* and *trans* isomers of octadecenoic and octadecadienoic acids in rats. Parameters investigated included the extent of uptake of ^{14}C-labeled *cis* and *trans* 18:1 and 18:2 by maternal plasma, placenta and fetal tissues. Maternal-fetal concentration gradients also were measured for oleic, elaidic, linoleic and linoelaidic acids. Finally, incorporation of label into placental and fetal tissue lipids, as well as biotransformation of *cis* and *trans* octadecenoic acids, were investigated.

MATERIALS AND METHODS

9-*trans* [1-^{14}C] Octadecenoic acid (elaidic acid), 55 mCi/mM, was purchased from Applied Science (Inglewood, CA) and 9-*trans*,12-*trans* [1-^{14}C] octadecadienoic acid (linoelaidic acid), 58 mCi/mM, was obtained from Rosechem (Los Angeles, CA). 9-*cis* [1-^{14}C] Octadecenoic acid (oleic acid), 57 mCi/mM, and 9-*cis*,12-*cis* [1-^{14}C] octadecadienoic acid (linoleic acid), 51

mCi/mM, were purchased from New England Nuclear (Boston, MA). Purity of the *cis* and *trans* fatty acids was determined by thin layer chromatography (TLC) on silver-nitrate-impregnated Silica Gel-60 plates using 5% acetone in toluene as the developing solvent (12). Radiopurity of *trans* and *cis* fatty acid isomers was greater than 98%.

On the 18th day of gestation, 300 μCi each of ^{14}C-labeled oleic, elaidic, linoleic, or linoelaidic acids were injected as albumin complexes into the jugular vein of rats. Each ^{14}C-labeled fatty acid was injected into 2 pregnant rats. One hr later, animals were anesthetized and blood was collected by open heart puncture. Fetus and placenta were removed and analyzed separately. Pregnant rats had 11-13 fetuses, and 3-4 fetuses were pooled for analysis of radioactivity. Brain and fetal body pools were examined separately. All tissues were frozen at -70 C until extraction for lipid analysis.

Total lipids (TL) from plasma and tissues were quantitatively extracted and purified by the Folch et al. method (13). Fractionation of TL was performed on Silica Gel-60 TLC plates using petroleum ether/ethyl ether/acetic acid (80:20:1, v/v) to obtain the percentage distribution of radioactivity in various lipid components (14). Areas indicated by brief exposure to iodine vapors were scraped directly into scintillation vials containing Aquasol (New England Nuclear, Boston, MA) and counted using a Beckman Liquid Scintillation Counter Model 800.

Fatty acid methyl esters (FAME) were obtained by methanolysis using 1% H_2SO_4 in methanol (14). The percentage distribution of radioactivity in FAME was analyzed with a Packard Radio-Gas Chromatograph Model 804, in combination with a Packard Gas Proportional Counter Model 894, fitted with a 6 ft. × 1/8 in. column packed with Silar 10C (Applied Science Laboratories, State College, PA) and run isothermally at 185 C. This instrument gives simultaneous peaks for mass and radioactivity.

Distribution of radioactivity in the carboxyl carbon relative to the intact fatty acid (% RCA) was determined. Individual pure FAME were obtained by preparative gas liquid chromatography (GLC) (15), hydrolyzed in 1 M KOH, and the resulting free fatty acids (FFA) were decarboxylated by the Schmidt method as described by Brady et al. (16).

RESULTS

Table I summarizes the percentage incorporation of radioactivity from injected oleic, elaidic, linoleic and linoelaidic acids into tissue lipids. About 0.2-1.0% of the injected isotopes was taken up by placenta, whereas, in general, 0.1-0.4% of the radioactivity was incorporated into fetal lipids. As compared to *cis* fatty acids, a greater incorporation of radioactivity was found in fetal tissues when *trans* fatty acids were injected into pregnant rats. Differences in the percentage incorporation of the dose by placenta following injection of *c,c*-18:2 and *t,t*-18:2 may reflect differences in uptake of the 2 isomers by maternal and placental tissues. Specific activities of plasma total lipids 1 hr after injection of *c,c*-18:2 and *t,t*-18:2 were 237,368 cpm/mg and 398,839 cpm/mg, respectively. Uptake of *c,c*-18:2 by maternal tissues may have occurred more readily than uptake of *t,t*-18:2, and, therefore, a corresponding decreased amount of incorporation of the injected dose of linoleic acid into placental and fetal tissues was observed.

Differences in specific activities of TL of maternal plasma, placental and fetal tissues indicated a decreasing concentration gradient for *cis* and *trans* fatty acids. Ratios of radioactivity of TL following injection of *cis* and *trans* octadecenoic acids were calculated and are diagrammed in Figure 1. Uptake of elaidic and oleic acids by placental tissue from maternal plasma was fairly similar. However, transfer

TABLE I

Percentage of Incorporation of the Dose[a,b]

Tracer injected	*c*-18:1	*t*-18:1	*c,c*-18:2	*t,t*-18:2
Placenta	0.47	0.45	0.17	1.04
Total fetus	0.09	0.38	0.10	0.32
Body	0.08	0.32	0.09	0.26
Brain	0.01	0.06	0.02	0.06

[a]Values expressed as the mean % incorporation of injected isotope for 2 rats in each case.

[b]Dose incorporated = Radioactivity in TL ÷ dose injected (μCi) × 100.

of oleic acid from placental to fetal tissue apparently was substantially less than transfer of elaidic acid as indicated by a much higher ratio of placental to fetal total radioactivity (4.95 vs 1.17 for *c*-18:1 and *t*-18:1, respectively).

Figure 2 illustrates differences in the ratios of radioactivity of TL following injection of *cis* and *trans* octadecadienoic acids. Placental uptake of linoleic acid from plasma was severalfold less than the uptake of linoelaidic acids (ratio of plasma to placental radioactivity of TL was 9.96 vs 3.41 for *c,c*-18:2 and *t,t*-18:2, respectively). In contrast, linoleic acid apparently crossed the placenta more readily than linoelaidic acid as reflected by a decreased ratio of radioactivity between placental and fetal TL (1.64 vs 3.26 for *c,c*-18:2 and *t,t*-18:2, respectively).

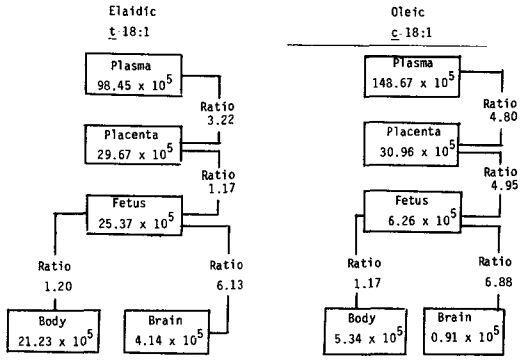

FIG. 1. Ratio of total radioactivity (cpm) following injection of *cis* and *trans* octadecenoate. Ratios were calculated by dividing total radioactivity of tissue TL.

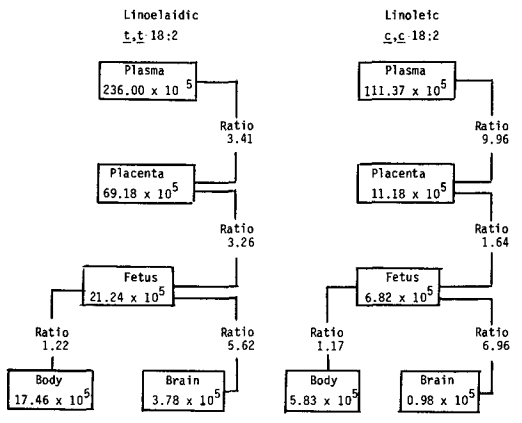

FIG. 2. Ratio of total radioactivity (cpm) following injection of *cis* and *trans* octadecadienoate. Ratios were calculated by dividing total radioactivity of tissue TL.

Table II shows the distribution of radioactivity in PL, cholesterol (C), FFA, TG and CE in maternal plasma and placental TL. In general, most of the radioactivity was associated with TG of the maternal plasma. However, even 1 hr after injection of oleic acid, a fairly high percentage of the total radioactivity was circulating as FFA. The percentage distribution of label was about equally divided between PL and TG of placental TL. Table III presents the distribution of radioactivity in fetal brain and the body. Irrespective of the label, 50-60% of the radioactivity in brain lipids was found in PL. Fetal body PL also contained the highest percentage of radioactivity. About 50% of the label in fetal tissue was associated with PL whereas 14-27% was incorporated into body TG.

One hr after injection of oleic acid, almost all of the radioactivity was associated with *c*-18:1 when total FAME were analyzed by radio-GLC (Table IV). In contrast, following injection of elaidic acid, differences in the pattern of distribution of label were found between plasma, placental and fetal FAME. In both plasma and placental TL, most of the radioactivity was present as *t*-18:1. However, in fetal tissues, ca. 30% of the total radioactivity was in palmitate (16:0). These results suggested that elaidic acid was oxidized and the resulting acetyl CoA was used for synthesis of 16:0 by fetal tissues.

One hr following injection of linoelaidic acid, again nearly all of the radioactivity was found as *t,t*-18:2 in plasma, placental and fetal tissue lipids. Following injection of linoleic acid, 94 and 100% of the radioactivity also was associated with *c,c*-18:2 in plasma and placental TL. However, ca. 23-29% of the label was associated with palmitate in fetal FAME. These findings suggested that fetal tissues oxidized linoleic acid and synthesized palmitate from the resulting acetyl CoA.

Distribution of radioactivity in the carboxyl carbon relative to the intact fatty acid (% RCA) has been used to establish pathways of metabolism (17). In this study, % RCA values approached 100% when oleic and linoelaidic acids were isolated from placental and fetal tissues. However, the distribution of radioactivity in palmitate isolated from fetal tissues following injection of *t*-18:1 and *c,c*-18:2 was similar to that predicted for de novo synthesis (17).

DISCUSSION

Transport of preformed lipids from the maternal circulation into the fetus has not been studied extensively because glucose is generally

TABLE II

Percentage of Distribution of Radioactivity in Maternal Plasma[a] and Placenta[b]

Tracer injected	*c*-18:1		*t*-18:1		*c,c*-18:2		*t,t*-18:2	
	Plasma	Placenta	Plasma	Placenta	Plasma	Placenta	Plasma	Placenta
PL	7.3	29.8 ± 1.6	4.3	41.7 ± 4.6	9.2	49.2 ± 0.5	4.8	39.7 ± 2.3
C	9.4	5.8 ± 1.3	23.4	16.3 ± 1.2	5.3	7.7 ± 0.7	2.9	4.3 ± 0.8
FFA	30.8	12.5 ± 1.7	8.7	5.1 ± 1.2	8.5	13.5 ± 1.7	6.2	6.2 ± 0.5
TG	37.7	49.8 ± 4.5	62.3	35.6 ± 6.0	74.3	27.4 ± 1.4	84.5	48.1 ± 2.0
CE	14.9	2.2 ± 0.2	1.3	1.3 ± 0.2	2.6	2.4 ± 0.9	1.6	1.8 ± 0.1

[a]Plasma values represent the mean of duplicate analyses.

[b]Values are expressed as the mean ± standard deviation of duplicate measurements of 3 pooled placental samples.

TABLE III

Percentage of Distribution of Radioactivity in Fetal Brain and Body[a]

Tracer injected	*c*-18:1		*t*-18:1		*c,c*-18:2		*t,t*-18:2	
	Brain	Body	Brain	Body	Brain	Body	Brain	Body
PL	64.2 ± 9.5	47.5 ± 5.3	50.5 ± 1.8	49.8 ± 1.2	57.6 ± 1.9	49.5 ± 11.9	62.8 ± 4.9	57.8 ± 2.4
C	6.9 ± 0.9	10.2 ± 1.5	28.0 ± 1.8	23.5 ± 1.0	11.0 ± 0.7	13.2 ± 2.3	7.5 ± 1.0	6.4 ± 0.7
FFA	12.9 ± 1.8	14.4 ± 1.6	10.0 ± 1.0	7.1 ± 2.8	15.2 ± 4.6	15.4 ± 2.6	9.1 ± 1.5	6.5 ± 0.5
TG	10.5 ± 4.5	19.7 ± 0.9	9.8 ± 1.9	18.0 ± 1.6	11.9 ± 4.0	13.7 ± 3.8	16.9 ± 7.4	26.6 ± 2.4
CE	5.5 ± 5.0	8.2 ± 2.1	1.8 ± 1.8	1.7 ± 0.2	4.3 ± 1.5	8.1 ± 4.1	1.6 ± 0.7	2.7 ± 0.3

[a]Values are expressed as the mean ± standard deviation of duplicate measurements of 3 pooled fetal brain and body samples.

TABLE IV

Percentage of Distribution of Radioactivity in FAME[a]

Fatty Acid	Plasma	Placenta	Brain	Body
		t-18:1 Injected		
16:0	16.7	4.4	36.1	33.3
t-18:1	83.3	95.6	63.9	66.6
		c-18:1 Injected		
c-18:1	100	100	100	100
		t,t-18:2 Injected		
t,t-18:2	100	100	100	100
		c,c-18:2 Injected		
16:0	5.9	–	29.0	23.3
18:0	–	–	9.5	–
c,c-18:2	94.1	100	61.5	76.7

[a]Values expressed as the mean of duplicate analyses of maternal plasma as well as pooled placenta and fetal tissues.

believed to be the major source of carbon used for lipid synthesis. The transport of fatty acids (saturated and *cis* isomers) across the placenta has been deduced from a variety of experimental studies. Fatty acids probably cross the placenta in the form of FFA and are later esterified in fetal tissues. Little is known, however, concerning the transport and further metabolic fate of *trans* fatty acids in the fetus. Thus, there is little information regarding metabolism of *trans* fatty acids during the critical and vulnerable period of intrauterine growth.

Following injection of labeled fatty acids, most of the radioactivity in maternal plasma was associated with TG. However, the label in placental lipids was found primarily in PL and TG. Differences in the distribution of radioactivity in plasma and placental lipids suggested that FFA introduced in the maternal circulation may have been taken up by the placenta and esterified into PL and TG in situ rather than reflect the transport of intact complex lipids. The distribution of radioactivity in placenta also probably reflected a contribution of label from the separate maternal and fetal circulations within placental tissue.

Similarly, differences in distribution of label between fractionated placental and fetal TL suggested that *cis* and *trans* octadecenoic and octadecadienoic acids crossed the placenta as FFA and were esterified primarily in PL of fetal tissues. In this study, PL contained the maximal radioactivity of the fetal lipids. Thus, these findings suggest that FFA were transported and subsequently esterified into complex lipids of the rapidly growing cell populations of the fetus.

The percentage distribution of radioactivity in fetal brain and body TL was similar, regardless of the fatty acid injected. Therefore, the lack of differences in the percentage distribution of label indicated that *cis* and *trans* octadecenoic and octadecadienoic acids were incorporated into fetal tissues in a similar fashion.

One hr after injection of linoelaidic acid, radioactivity in plasma, placental and fetal tissue lipids was associated only with *t,t*-18:2, suggesting that acetyl CoA produced by oxidation of *t,t*-18:2 was not used to form 16:0. However, radioactivity in cholesterol indicates that radioactive acetyl CoA was indeed formed, perhaps from a nonhomogenous pool of acetyl CoA. One hr following injection of elaidic acid, most of the label in the circulating plasma and placenta also was associated with *t*-18:1. However, ca. 30% of the total radioactivity in fetal tissue was in palmitate. These results indicated that elaidic acid was oxidized and contributed acetyl CoA to de novo synthesis of palmitate. Percentage RCA data confirmed these conclusions. Thus, transfer and subsequent oxidation of elaidic acid by fetal tissues seen in our study may explain results of earlier studies (6,7,11) reporting little placental transfer of elaidic acid.

Findings of this investigation have demonstrated a difference in the fetal metabolism of elaidic and linoelaidic acids. Furthermore, these findings suggest that elaidic acid can serve as a potential source of energy for the developing fetus. In the developing brain, earlier studies by Karney and Dhopeshwarkar (18) demon-

strated that, although elaidic acid was preferentially incorporated into the 1-position of brain lecithin, linoelaidic acid was esterified mainly at the 2-position. We are now examining whether fetal tissue also differentiates between the *trans* octadecenoic and octadecadienoic isomers during fetal lecithin synthesis.

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Effects of Dietary *trans* Acids on the Biosynthesis of Arachidonic Acid in Rat Liver Microsomes

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ABSTRACT

Effects of dietary *trans* acids on the interconversion of linoleic acid was studied using the liver microsomal fraction of rats fed a semipurified diet containing fat supplements of safflower oil (SAFF), hydrogenated coconut oil (HCO) at 5 and 20% levels or a 5% level of a supplement containing 50.3% linolelaidic and 24.3% elaidic acids devoid of *cis,cis*-linoleic acid (TRANS). Growth rate was suppressed to a greater extent with the animals fed the 20% than the 5% level of the HCO-supplemented diets and still further by the TRANS diet compared to the groups fed the SAFF diets. Food intake was greater in the groups fed the HCO than the SAFF-supplemented diets, demonstrating the marked effect of an essential fatty acid (EFA) deficiency on feed efficiency. In contrast to an EFA deficiency produced by the HCO supplement, which stimulated the in vitro liver microsomal biosynthesis of arachidonic acid, diets containing the TRANS supplement exacerabated the EFA deficiency and depressed 6-desaturase activity of the liver microsomal fraction. The liver microsomal fraction of the animals receiving this supplement also was more sensitive to fatty acid inhibition of the desaturation of linoleic acid than those obtained from animals fed either the SAFF or HCO diets. It is suggested that dietary *trans* acids alter the physical properties of the 6-desaturase enzyme system, suppressing its activity, which increases the saturation of the tissue lipids and, in turn, the requirement for EFA or polyunsaturated fatty acids.

INTRODUCTION

The inhibitory effect of linolelaidic and elaidic acids on 6-desaturase activity, which is the key reaction in the regulation of the conversion of linoleic to arachidonic acid (1,2), has been well demonstrated by in vitro experiments with the liver microsomal fraction of rats fed a fat-deficient diet by Brenner and Peluffo (3). Nutritional experiments with rats also have shown that linolelaidic acid inhibits the conversion of oleic to 5,8,11-eicosatrienoic acid and linoleic to arachidonic acid (4-7). Elaidic acid also appears to have a similar effect, but to a relatively minor extent compared to linolelaidic acid (4,5). Linolelaidic acid itself is not converted in vivo to polyunsaturated fatty acids as shown in nutritional studies (4-7), and as recently demonstrated by radioactive tracer experiments in rat brain by Karney and Dhopeshwarkar (8). However, linolelaidic acid, as well as *cis,trans* isomers of linoleic and elaidic acids, are incorporated into the lipids of most tissues (4-11), and are readily metabolized (12).

In order to further determine the nutritional effects of *trans* acids, a study was made of the effects of an EFA deficiency and its exacerbation by feeding rats a diet containing a fat supplement of *trans* acids devoid of *cis,cis*-linoleic acid, as the sole source of fat in the diet on the interconversion of linoleic acid by liver microsomes.

MATERIALS AND METHODS

Experimental

Animals. Weanling male Sprague-Dawley rats (Dan Rolfsmeyer Co., Madison, WI) were divided into 5 groups of 10 animals and fed a basic fat-free diet supplemented with 5 or 20% by weight of safflower oil (SAFF), groups I and II, respectively; 5 or 20% of hydrogenated coconut oil (HCO), groups III and IV, respectively; or 5% of an ethyl ester concentrate of *trans* acids containing 50.3% linolelaidic acid (*t,t*-18:2) and 24.3% (*t*-18:1) devoid of *cis,cis*-linoleic acid (TRANS), group V. The diets (Table I) were made isocaloric by adjusting the relative amounts of fat, carbohydrate and fiber. The composition of the fat supplements and the contribution of each fatty acid to the dietary calories are shown in Table II.

The animals were weighed at 2-day intervals, food intake was also measured and daily consumption as well as feed efficiency determined. After the growth rate reached a plateau, the animals in each group were killed under a light ether anesthetic by withdrawal of blood from the retroocular plexes.

Preparation of liver microsomes. Freshly excised livers were perfused with saline and homogenized in a Potter-Elvehjem homogenizer in 2 vol of a solution containing 0.25 M sucrose, 5 mM $MgCl_2$, 0.15 M KCl, 1.5 mM GSH and 50

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TABLE I

Diet Composition

	5% Fat diet (% by wt)	20% Fat diet (% by wt)
Casein (vitamin test)	22.50	22.50
L-cystine[a]	0.20	0.20
Wesson salt mixture plus $ZnCl_2$ and $MnSO_4 \cdot H_2O$[b]	4.03	4.03
Choline mix[c]	1.00	1.00
Vitamin mix[d]	1.00	1.00
Cellulose (Alphacel)	10.25	29.00
Sucrose	56.02	22.27
Fat	5.00	20.00

[a]L-Cystine is added to the diet to bolster the level of sulfur-containing amino acids.

[b]Wesson salt mixture does not contain zinc or manganese, hence these elements are added to the mix as follows: 0.60 g of $ZnCl_2$ and 0.90 g of $MnSO_4 \cdot H_2O$/200 g of salt mixture.

[c]Choline mix consists of 22% choline dihydrogen citrate in vitamin test casein.

[d]One kg of the vitamin mix contains: 2.5 g thiamine HCl, 2.5 g riboflavin, 9.0 g nicotinic acid, 9.0 g calcium pantothenate, 2.0 g pyridoxine HCl, 4.0 g cyanobalamin (B_{12}), 7.5 g *p*-aminobenzoic acid, 0.1 g folic acid, 0.02 g biotin, 20.0 g *meso*-inositol, 0.5 g menadione (vitamin K), 943.0 g vitamin test casein. Fat and vitamins A, D and E are mixed into the diet daily and stored at 0 C overnight. Vitamin D_2, 5.0 mg; retinol acetate, 6.9 mg; α-tocopherol acetate, 300 mg/kg of diet.

mM of potassium phosphate buffer pH 7.0. Homogenates were centrifuged at 800 × g for 10 min to remove cell debris, then at 10,000 × g for 20 min. The microsomal fraction was recovered by centrifuging the 10,000 × g supernatant at 100,000 × g for 1 hr. The pellet was suspended in a solution containing 5 mM $MgCl_2$, 0.15 M KCl, 1.5 mM GSH and 50 mM potassium phosphate buffer (pH 7.0) to give a final concentration of 20 mg/ml protein, determined by the method of Lowry et al. (13).

Incubation conditions. ATP, CoASH, malonyl-CoA, NADH and bovine serum albumin containing less than 0.005% fatty acid were purchased from Sigma Chemical Co., St. Louis, MO, GSH from P-L Biochemical, Inc., Milwaukee, WI, and [1-^{14}C]linoleic acid (50 μCi/μmol) from New England Nuclear, Boston, MA. The [1-^{14}C]linoleic acid was purified by thin layer chromatography, converted to the ammonium salt and bound to bovine serum albumin (14). All incubations were carried out at 37 C in a total vol of 2.0 ml. For measurement of desaturase activity, each incubation was conducted for 10 min in the medium containing the following: 10 μmol of $MgCl_2$, 0.3 mmol of KCl, 3 μmol of GSH, 10 μmol of ATP, 0.6 μmol of CoA, 2.5 μmol of NADH, 100 μmol of potassium phosphate buffer, pH 7.0, 200 nmol of radioactive linoleic acid and 5 mg microsomal protein in 0.001% Triton X-100. The same conditions were used for determination of desaturation chain elongation reactions except that 0.6 μmol of malonyl CoA was added to the incubation mixture.

Incubations were stopped by the addition of 10 ml of dimethoxypropane (DMP) containing 200 μl of concentrated HCl to 1 ml of the incubation medium. After a reaction time of 20 min to allow for conversion of the water to methanol and acetone, these solvents and the excess DMP were evaporated in a stream of nitrogen at room temperature and the lipid was interesterified with methanol as described by Shimasaki et al. (15).

Radioactivities of the methyl esters were determined on fractions isolated by gas liquid chromatography using an Aerograph Model 600-D gas chromatograph equipped with a 9:1 splitter and a 12' × 0.125" id column packed with 10% Silar 10C on 100-200 mesh Gas-Chrom Q at 210 C with a flow rate of N_2 of 20 cc/min. Fractions corresponding to each fatty acid ester (peaks in the chromatogram) were collected in glass tubes attached directly to the outlet of the splitter, coincident with their detection by the flame detector, and transferred to scintillation counting vials by washing the tubes with 15 ml of scintillation fluid (5.5 g of Permablend I/liter of toluene). Radioactivity was counted in a Packard Model 3310 scintillation spectrometer. Recovery of radioactivity by this technique of collection was ca. 75%. The activity of 6-desaturase, determined independently, was calculated from the counts of 18:3 corrected for background. The activities of 6-desaturase, chain elongation and

TABLE II

Fatty Acid Composition and Caloric Level of Dietary Fats[a]

	SAFF supplement			HCO supplement				TRANS supplement		
		Group I (5% wt)	Group II (20% wt)			Group III (5% wt)	Group IV (20% wt)			Group V (5% wt)
Fatty[b] acids	Fat (% wt)	Dietary calories (%)	Dietary calories (%)	Fatty acids	Fat (% wt)	Dietary calories (%)	Dietary calories (%)	Fatty acids	Fat (% wt)	Dietary calories (%)
16:0	9.6	1.2	4.8	10:0	5.7	0.4	1.76	16:0	18:1	2.3
18:0	3.6	0.5	2.0	12:0	45.2	5.9	23.6	18:0	7.3	0.9
18:1	13.4	1.7	6.8	14:0	16:8	2.5	10.0	*t*-18:0	24.3	3.0
18:2	73.4	9.2	36.8	16:0	8.8	1.4	5.6	*tt*-18:2	50.3	6.3
				18:0	13:8	1.9	7.6			
				18:1	1.7	0.3	1.16			
				18:2	0.1-0.5					

[a]SAFF = safflower oil; HCO = hydrogenated coconut oil; TRANS = ethyl ester-concentrate of *trans* acids.

[b]Shorthand designation; number before colon = chain length; number after colon = number of double bonds, *t* = *trans* double bond.

5-desaturase in the overall reaction were determined simultaneously from the total counts of the products corrected for background, the proportion of the counts in the 20:3 + 20:4 acids, and the 20:4 fraction, respectively. The products of the reactions (18:3 and those of chain elongation after a short lag period) under the assay conditions just described were proportional to protein concentrations in excess of 5 mg in the incubation mixture and a reaction time of ca. 15 min.

Fatty acid composition. The fatty acid composition of liver microsomal lipid was determined on methyl esters prepared by interesterification with HCl as a catalyst (15) using a Hewlett Packard Model 5840A gas chromatograph. This analysis also was carried out with a 12' x 0.125" id column packed with Silar 10C on 100:200 mesh Gas-Chrom Q at 200-250 C programmed at 2.0 C/min with a flow rate of nitrogen of 10 cc/min.

The highly purified fatty acid standards (>99%) and the ethyl ester concentrate of *trans* acids which contained 50.3% linolelaidic acid (*t,t*-18:2) and 24.3% elaidic acid (*t*-18:1) (Table II) were obtained from Nu-Chek-Prep, Inc., Elysian, MN.

RESULTS

Weight gains of the animals in each group (Fig. 1) showed that although there was no difference in the growth rates of the 5 and 20% SAFF groups, that of the 20% HCO group was significantly lower than the 5% HCO group. The TRANS dietary regimen gave an even greater suppression of growth rate than the 20% HCO diet as also shown in Figure 1. Measurement of food intake of the animals in each group (Table III) showed that, in spite of differences in growth rate, the animals of the HCO-supplemented groups consumed more food than those of the SAFF groups. The animals in the TRANS group consumed the least food and their growth was suppressed the most. Accordingly, feed efficiency was highest in the SAFF group and greatly diminished in the other groups. The difference in growth rate between the 5 and 20% HCO groups was also reflected by corresponding differences in feed efficiency.

The fatty acid composition of the liver microsomal lipid of the animals in each group reflected generally that of the dietary fat as shown in Table IV. The SAFF supplement, which contained ca. 70% linoleic acid, supplied more than an adequate amount of this fatty acid in the diet to meet the requirement for essential fatty acids even at the 5% dietary

level. Evidence to this effect was that the levels of arachidonic acid in the microsomal lipid of the 5 and 20% SAFF groups were essentially the same, ca. 32%, in spite of the fact that the diet of the 20% SAFF group contained a much larger amount of linoleic acid (Table II). The fatty acid composition of the liver microsomal lipid of these animals (groups I and II) exhibited the normal pattern for animals receiving a diet adequate in essential fatty acids (EFA). The liver microsomal lipid of the animals receiving the HCL supplements exhibited a fatty acid composition typical of an EFA deficiency. Compared to the SAFF groups, the levels of 16:1 and 18:1 were elevated. Those of linoleic and arachidonic acid were decreased and there was an appreciable formation of 20:3, giving a high triene-to-tetraene ratio in the microsomal lipid of the HCO groups (III and IV). The pattern of these changes was more pronounced in the 5% HCO group than the 20% group because the hydrogenated coconut oil

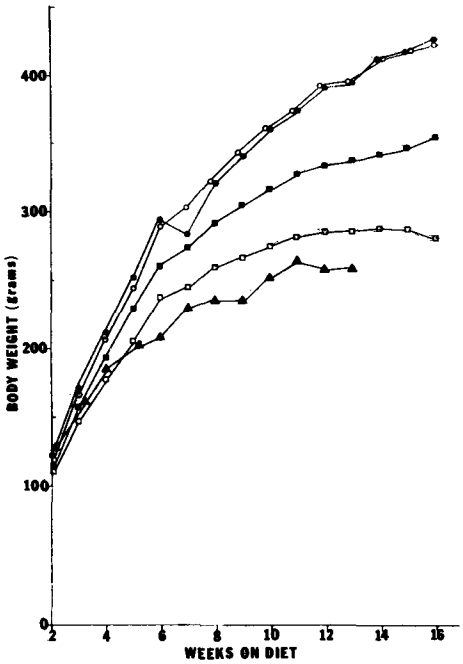

FIG. 1. Growth rate of rats from weaning fed a fat-free diet supplemented with: 5% or 20% safflower oil, groups I and II, respectively, open and solid circles; 5% hydrogenated coconut oil, group III, solid squares; 20% hydrogenated coconut oil, group IV, open square; 5% ethyl ester concentrate of *trans* acids, group V, solid triangles. At 14 weeks, the weights (M ± SD) of the animals of each group were as follows: I, 411 ± 16; II, 411 ± 32; III, 342 ± 22; IV, 286 ± 25 and V, 260 ± 22. III was significantly greater than IV ($P < .001$) and IV was significantly greater than V ($P < .100$).

TABLE III

Feed Efficiency[a]

Age (weeks)	Group[b] 5% HCO			20% HCO			5% SAFF			20% SAFF			5% TRANS		
	Food intake			Food intake			Food intake			Food intake			Food intake		
	Daily	Total[a]	Feed[a] eff.	Daily	Total	Feed eff.	Daily	Total	Feed eff.	Daily	Total	Feed eff.	Daily	Total	Feed eff.
3	6 ± 1.9[c]	6 ± 1.9	–	8.1 ± 3.9	8.1 ± 3.9	–	7.2 ± 1.9	7.2 ± 1.9	–	8.2 ± 1.3	8.2 ± 1.3	–	5.0 ± .71	5.0 ± .71	–
4	13 ± 1.4	67 ± 4.9	1.0 ± .07	14.9 ± 3.3	73.6 ± 19	.98 ± .24	11.4 ± 1.7	73 ± 15	1.4 ± .12	19.8 ± .45	80 ± 8.3	1.2 ± .07	9.6 ± 2.0	62 ± 1.6	1.4 ± .14
5	17 ± 4.2	171 ± 17	.68 ± .05	18.5 ± 1.2	193 ± 30	.61 ± .10	15.2 ± 1.6	169 ± 28	.87 ± .04	15.0 ± 2.8	187 ± 21	.78 ± .03	14 ± 1.4	151 ± 9.2	.84 ± .08
6	19 ± 3.8	287 ± 44	.57 ± .07	20 ± 4.8	321 ± 51	.46 ± .07	16.2 ± 1.6	273 ± 47	.71 ± .09	16.2 ± 1.5	300 ± 28	.62 ± .02	15.6 ± .89	252 ± 11	.64 ± .06
7	18 ± 2.9	422 ± 59	.46 ± .05	21.4 ± 4.4	462 ± 77	.39 ± .06	17.8 ± 1.6	411 ± 53	.59 ± .04	18.4 ± 3.2	423 ± 37	.56 ± .01	15.8 ± 1.9	293 ± 146	.54 ± .08
15	22 ± 1.9	1653 ± 160	.20 ± .02	23.8 ± 4.2	1818 ± 308	.16 ± .02	17.0 ± 2.8	1466 ± 157	.26 ± .01	18.4 ± 6.5	1441 ± 121	.27 ± .01	17 ± 1.2	1254 ± 96	.21 ± .01
16	20 ± 2.9	1799 ± 175	.18 ± .02	21.2 ± 6.4	1979 ± 345	.15 ± .02	16.8 ± 1.3	1589 ± 167	.25 ± .01	17.4 ± 2.9	1540 ± 152	.26 ± .02	15.2 ± 1.3	1358 ± 101	.19 ± .01
19	19 ± 2.3	2339 ± 211	.15 ± .01	19.8 ± 3.9	2539 ± 436	.12 ± .02	15.8 ± 1.1	1938 ± 193	.22 ± .01	15.6 ± 2.07	1902 ± 167	.22 ± .01	–	–	–
20	19 ± 2.7	2471 ± 221	.14 ± .01	23 ± 4.5	2705 ± 467	.11 ± .02	17.2 ± 18.4	2057 ± 203	.21 ± .01	17.2 ± 1.6	2020 ± 179	.21 ± .01	–	–	–

[a]Grams gained/grams of feed consumed; no allowance made for amount of milk consumed before weaning.

[b]HCO = hydrogenated coconut oil; SAFF = safflower oil; TRANS = trans acid concentrate.

[c]M ± SD, 5 animals; feed efficiency of 5% HCO vs 20% HCO significant, $P > .025$ at 20 weeks; feed efficiency of 5% HCO vs 20% SAFF significant, $P < .001$ at 20 weeks; total food intake of 5% HCO vs 20% SAFF significant, $P < .01$ at 20 weeks.

TABLE IV

Fatty Acid Composition of Liver Microsomal Lipid (% wt)

Dietary group[a]	I 5% SAFF	II 20% SAFF	III 5% HCO	IV 20% HCO	V 5% TRANS
16:0[b]	18.7 ± 1.1[c]	15.0 ± 0.5	19.6 ± 0.9	17.3 ± 1.0	15.9 ± 0.2
16:1	3.0 ± 0.7	0.7 ± 0.1	10.3 ± 0.1	4.1 ± 0.6	12.3 ± 1.5
18:0	14.3 ± 0.5	22.6 ± 0.8	18.9 ± 1.1	25.0 ± 0.6	13.0 ± 0.9
18:1	7.3 ± 1.0	4.6 ± 0.1	20.7 ± 1.5	15.6 ± 0.3	29.3 ± 1.9
t-18:1					2.2 ± 0.9
18:2	10.1 ± 1.1	16.1 ± 0.5	2.7 ± 0.1	4.3 ± 0.3	5.3 ± 0.2[d]
20:3ω9			14.0 ± 0.2	7.3 ± 0.2	5.1 ± 0.7
20:4	31.2 ± 1.8	32.2 ± 0.2	6.5 ± 0.7	17.0 ± 0.7	3.8 ± 0.1
22:4	4.4 ± 0.5	3.0 ± 0.4	1.0 ± 0.1	2.5 ± 0.2	0.6 ± 0.1
20:3/20:4			2.2	0.43	1.3

[a]SAFF = safflower oil; HCO = hydrogenated coconut oil; TRANS = concentrate of ethyl linoleate (Table II); see Materials and Methods for additional details of dietary regimens.

[b]Shorthand designation of fatty acids; number before colon = chain length; number after colon = number of double bonds.

[c]M ± SD, n = 4.

[d]Contains a mixture of positional and geometric isomers.

supplement contained a small amount of linoleic acid (Table II) which provided a significant amount of this fatty acid in the diet, particularly in the animals fed at the 20% level, group IV. Accordingly, the levels of 18:2 and 20:4 were higher in 20% than the 5% HCO group and the effect on the composition of the other fatty acids was not so great (Table IV) giving a relatively low triene-tetraene ratio indicative of only a borderline EFA deficiency in this group, IV. However, in spite of the fact that the EFA deficiency was only borderline, the growth rate of the animals of this group was suppressed to a greater degree than that of the animals of the 5% HCO group (Fig. 1).

The TRANS supplement was devoid of linoleic acid (Table II), hence the levels of linoleic and arachidonic acids were very low in the animals of group V, and the severity of the EFA deficiency was greater in the animals of this group than those of groups III and IV. The triene-tetraene ratio was elevated in this group, indicative of an EFA deficiency, but it was not as high as in the 5% HCO group. The triene-tetraene ratio does not give a true indication of the EFA deficiency in animals fed linolelaidate because this acid inhibits the conversion of oleic to 20:3 and 18:2 to 20:4 as shown in previous work (4-7). Accordingly, the level of arachidonic acid in the liver microsomal lipid of the animals of this group was very low compared to that of the SAFF animals (Table IV). Likewise, the level of 20:3 was lower in liver microsomal lipid of these animals than in those of the HCO groups (III and IV). The levels of 18:1 and 16:1 were elevated in this group (V) even above those of the HCO groups, which were already high compared to those in the animals of the SAFF groups. The positional and geometric isomers of the 18:2 acids were not identified in the analysis of the liver microsomal lipid of this group, but this fraction should consist mainly of linolelaidate inasmuch as the dietary fat was devoid of *cis,cis* linoleate. This fraction also might contain some 5,9-18:2 as a product of the desaturation of elaidate (16,17), as well as 6,9-, 5,8- and 8,11-isomers found in EFA-deficient animals (18). However, the actual composition of this fraction will have to await further, detailed analyses. Regardless, the data in Table IV show that the dietary regimens produced animals having liver microsomal lipid of widely different fatty acid compositional patterns; namely, a normal pattern, groups I and II, 2 levels of a typical EFA deficiency, groups III and IV, and a third pattern characteristic of an EFA deficiency complicated by an effect of dietary *trans* acids, group V.

The effect of the dietary regimens on the interconversion of linoleic acid by liver microsomes in vitro is shown in Table V. These analyses showed that the simple EFA deficiency developed in groups III and IV by feeding the HCO supplement produced a marked elevation in the activity of the 6-desaturase chain elongation enzyme system by comparison with groups I and II. In these experiments, the activities of the chain elongation and 5-desaturase enzyme systems are dependent on substrates produced in the interconversion of linoleic acid, and do not

TABLE V

Liver Microsomal Enzyme Activities (nmol/min/mg protein)

Dietary group[a]	I 5% SAFF	II 20% SAFF	III 5% HCO	IV 20% HCO	V 5% TRANS
6-Desaturase[b]	0.315 ± .078[c]	0.436 ± .010	0.661 ± .004	0.644 ± .028	0.238 ± .004
6-Desaturase	0.343 ± .076	0.470 ± .016	0.654 ± .031	0.686 ± .055	
Chain elongation	0.234 ± .049	0.305 ± .020	0.451 ± .013	0.470 ± .050	
5-Desaturase	0.062 ± .009	0.121 ± .001	0.147 ± .007	0.137 ± .018	

[a]SAFF = safflower oil; HCO = hydrogenated coconut oil; TRANS = concentrate of ethyl linolelaidate, (Table II), see Materials and Methods for additional details of dietary regimens.

[b]Determined independently.

[c]M ± SD (n = 4). Group I vs group II, 6-desaturase $P < .025$; group I vs group V, 6-desaturase $P < .01$; group II vs group IV, 6-desaturase $P < .001$.

represent maximal reaction rates.

In contrast to groups III and IV, the 6-desaturase activity of the liver microsomal fraction of the TRANS group (V) was significantly lower than that of the SAFF groups. The suppression of 6-desaturase activity in the animals of the TRANS group (V) was further demonstrated by comparison of different concentrations of ATP on the activity of this enzyme system in the liver microsomal fraction of animals from the 3 different dietary groups as shown in Figure 2. These experiments demonstrated that, while an EFA deficiency of the type produced by depriving the animals of adequate linoleate elevated 6-desaturase activity (group IV), that produced by the effect of the TRANS supplement (group V) suppressed the activity of this enzyme system. This experiment also showed that the lower activity of the microsomal fraction of the TRANS group was not due to impaired activation of substrate.

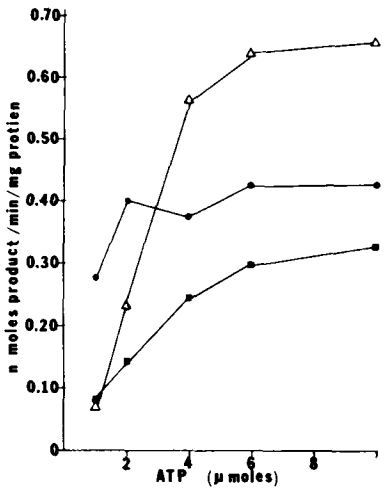

FIG. 2. Effect of ATP concentration on the rate of desaturation of linoleic acid in vitro by rat liver microsomal fractions obtained from rats fed diets supplemented with: 20% hydrogenated coconut oil, group IV, open triangles; 20% safflower oil, group II, solid circles; 5% ethyl ester concentrate of *trans* acids, group V, solid squares.

A comparison of the inhibitory effects of fatty acids on the desaturation of linoleic acid by the liver microsomal fraction of animals from the different dietary regimens is shown in Figure 3. In these experiments, the substrate consisted of a mixture of 20 or 200 nmol of palmitic, stearic, oleic, elaidic or linolelaidic acids plus 200 nmol of [1-^{14}C]linoleic acid. These experiments showed that inhibition of 6-desaturase activity by these fatty acids, particularly in the palmitic and stearic acids, was much greater with the liver microsomal fraction obtained from the animals fed the TRANS supplement than of those fed the HCO or SAFF supplements.

DISCUSSION

In this study, the in vitro experiments showed that 6-desaturase activity of liver microsomal preparations was enhanced in an EFA deficiency produced by the HCO diet but was suppressed in animals fed the TRANS-supplemented diet, although this diet also produced an EFA deficiency. These experiments are in accord with the nutritional effects of these dietary supplements inasmuch as oleate is readily converted to 20:3 in an EFA deficiency produced by feeding HCO-supplemented diets, and the reaction is suppressed in animals in which an EFA deficiency is produced upon feeding diets containing supplements of linolelaidic acid (4-7). Although elaidic acid also suppresses the conversion of oleic to 20:3, its effect is minor compared to linolelaidic acid as indicated in previous studies (4,5). Thus, the effect of the TRANS dietary supplement

appears to primarily result from its content of linolelaidic acid.

As a result of the suppression of 6-desaturase activity in the animals fed diets containing linolelaidate, the level of 20:3, which accumulates to high levels in an EFA deficiency, is relatively low in the liver microsomal fraction of these animals. As shown here and in previous work (4-7), the level of *cis* 18:1 is elevated simultaneously with a decrease in 20:3 in animals fed linolelaidate. Guo and Alexander (16) have shown that dietary linolelaidic acid contributes to the pool of *cis* 18:1 via de novo synthesis from acetate produced in its catabolism as opposed to a pathway involving biohydrogenation. Thus, the elevated level of 18:1 indicates that linolelaidate does not inhibit de novo fatty acid synthesis or impair 9-desaturase activity in animals fed diets containing *trans* fatty acids.

It appears paradoxical that linolelaidic acid inhibits 6-desaturase activity but enhances the formation of 18:1 in vivo, that is, 9-desaturase activity, particularly as the activity of both enzymes are elevated in an EFA deficiency produced by feeding either a fat-free or an HCO-supplemented diet (19-21). However, as pointed out by Peluffo et al. (22) and demonstrated by others (23,24), the 6- and 9-desaturase enzyme systems respond differently to a number of dietary and hormonal stimuli. Several investigators (25-27) have suggested that desaturase activity is regulated, in part, by the synthesis of enzyme protein, whereby enzyme concentration is a factor. In accord with this hypothesis, it has been shown that the level of 9-desaturase is elevated in an EFA deficiency (25). The elevation of desaturase activity in animals with an EFA deficiency has been studied extensively by Peluffo et al. (22) and by Holloway and Holloway (25). The former investigators found that 6-desaturase activity correlated with changes in the triglyceride-phospholipid ratio. Both groups of investigators considered that the increase in desaturase activity might be related to membrane fluidity in which enzyme activity was increased to maintain the ratio of unsaturated to saturated fatty acids. However, unsaturation of the tissues is decreased by the TRANS supplement more than by the HCO dietary fat. Thus, *trans* acids apparently have a direct effect on the 6-desaturase enzyme system. Moreover, it seems unlikely that the effect of *trans* acids on 6-desaturase can be explained on the basis of decreased protein synthesis inasmuch as 9-desaturase activity apparently is elevated when these acids are fed as the sole source of fat in the diet.

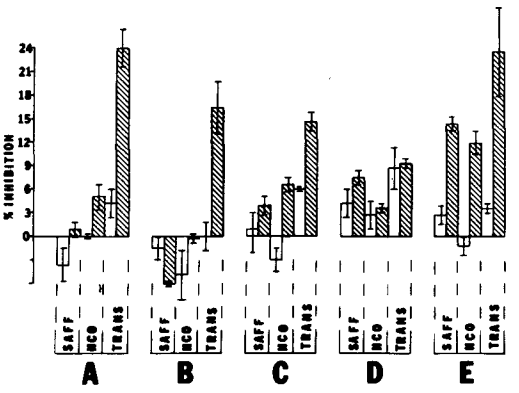

FIG. 3. Inhibition of the desaturation of linoleic acid by liver microsomal fractions obtained from rats fed diets supplemented with safflower oil (SAFF), hydrogenated coconut oil (HCO) or a concentrate of *trans* acids (TRANS) with A, palmitic acid (16:0); B, stearic acid, (18:0); C, oleic acid (*c*-18:1); D, elaidic acid (*t*-18:1); E, linolelaidic acid (*t,t*-18:2). Open bars, 20 nmol, and hatched bars, 200 nmol of each acid with 200 nmol of [1-^{14}C]linoleic acid. Inhibitions were significant for 200 nmol of all fatty acids with the microsomal fraction of the TRANS group as follows: 16:0, $P < .001$; 18:0, $P < .025$; *c*-18:1, $P < .001$; *t*-18:1, $P < .001$; *t,t*-18:2, $P < .005$.

Brenner and Peluffo (3) showed that both linolelaidate and elaidate exhibited an inhibitory effect in vitro on the desaturation of linoleic acid by the rat liver microsomal fraction of EFA-deficient animals. Recently, Mahfouz et al. (28) demonstrated that *trans* monounsaturated acids inhibit 9- as well as 5- and 6-desaturase activity of the liver microsomal fraction of EFA-deficient rats in in vitro experiments. The in vitro experiments reported herein show that *trans* acids inhibit 6-desaturase activity of the liver microsomal fraction of animals fed a diet containing adequate linoleate (SAFF group) and animals fed the TRANS supplement, devoid of EFA and presumably highly deficient in EFA as well as animals deprived of adequate linoleate by feeding hydrogenated coconut oil. However, of particular significance is the observation that the liver microsomal fraction of the animals receiving the TRANS-supplemented diet are more sensitive to the inhibitory effects of fatty acids than that of either the HCO or SAFF groups. Thus, it is apparent that these fatty acids alter the physical properties of the 6-desaturase enzyme system in some manner. It has been shown that acyl desaturases have a lipid requirement (29-31), hence the effect on the 6-desaturase system by *trans* acids might result from an alteration of the composition of the lipid cofactor.

The nutritional data indicate that low growth rate in the animals of the HCO and TRANS groups is related to low feed efficiency, which in turn is associated with the EFA deficiency in these groups of animals. The fact that the growth rate was lower in the animals of the 20% than in the 5% HCO group indicated that the high level of saturated dietary fat increased the EFA requirement in this group. However, the high level of saturated fat did not inhibit the conversion of linoleic to arachidonic acid as the animals of the 20% HCO group exhibited only a borderline EFA deficiency as evidenced by the levels of linoleate and arachidonate in the microsomal tissue lipid. The increased requirement of EFA in diets containing high levels of saturated fat is generally explained on the basis of the importance of dietary linoleate for the utilization of saturated fatty acids (32-35). However, Privett et al. (36,37) observed that both fish oils and concentrates of eicosapentaenoic and docosahexaenoic acids which did not cure an EFA deficiency stimulated the growth of EFA-deficient animals. Moreover, Kurata and Privett (38) observed that dietary Menhaden oil inhibited the conversion of linoleate to arachidonate which apparently was due to the suppression of 6-acyl desaturase activity by eicosapentaenoic and docosahexaenoic acids. Thus, the nutritional effects of *trans* acids probably is largely due to inhibition of the 6-acyl desaturation system which increases the saturation of the tissue lipids and, in turn, the requirement for EFA or polyunsaturated fatty acids.

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Dynamic Lipid Changes in Rapidly Proliferating Hepatic Smooth Endoplasmic Reticulum during Acute Dexamethasone Treatment of Adrenalectomized Rats

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ABSTRACT

The regulation by the cell of subcellular membrane components is dependent on a highly complex balance of nutritional, hormonal and metabolic events. We have characterized the lipid components of the endoplasmic reticulum (ER) of the liver of adrenalectomized (ADX) rats and the response of these membrane components to glucocorticoid administration. Membrane microviscosity as measured by fluorescence depolarization of 1,6-diphenylhexatriene (DPH) was measured and correlated with lipid composition and content of the membranes. In the ADX rat, a significant increase in membrane microviscosity of the smooth endoplasmic reticulum (SER) was observed and this was accompanied by an increase in the cholesterol content/mg protein and a decrease in the phospholipid content/mg protein. A change in the fatty acyl chain composition is observed with a significant increase in the mole percentage of arachidonic acid (20:4) and an accompanying decrease in saturated fatty acids. Within 2-6 hr of dexamethasone administration, a decrease in membrane microviscosity is observed that returns this value to one similar to that for normal control animals. Both the cholesterol and the phospholipid contents/mg protein are likewise restored to levels similar to that for control animals beginning at the 2-hr time point. The arachidonic acid and saturated fatty acid content of the constituent phospholipids do not begin to return to values similar to those for control animals until 6 hr after dexamethasone administration. From these experiments, we can conclude that glucocorticoids play a significant regulatory role in determining the lipid properties of rat hepatic microsomal membranes.

INTRODUCTION

It is well documented that cell membranes are composed of predominantly a lipid bilayer that is fluid in nature with interdispersed proteins (1). The lipid bilayer in animal cells contains a wide variety of chemically distinct phospholipids as well as cholesterol arranged in a fashion to meet the functional requirements of the membrane. The regulation by the cell of these lipid membrane components is highly complex since it must involve responses to nutritional, hormonal and metabolic stimuli balanced with maintaining the functional integrity of the membrane. Studies of a wide variety of membrane systems indicate that physical characteristics of membrane lipids have modulating effects on transport functions, enzyme activity and hormone-receptor interactions (2-5).

A number of different approaches have been used to determine the physical characteristics of membrane lipids including electron-spin resonance techniques, differential scanning calorimetry and fluorescence polarization measurements. With regard to fluorescence polarization measurements, the fluorescent probe 1,6-diphenylhexatriene (DPH) has been employed as a probe in membrane studies for determining membrane lipid fluidity and this has been reviewed recently (6). The probe is distributed evenly in the hydrocarbon chain of the lipid bilayer and the derived microviscosity is a weight average of all lipid domains. Three prominent determinants of membrane microviscosity are the cholesterol/phospholipid ratio, the degree of unsaturation of the phospholipid acyl chains, and phospholipid polar head-groups (7-10).

Numerous morphological and biochemical studies have indicated that both rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) of rat liver undergo changes in form and amount in response to changes in nutritional and hormonal status (11-15). Indeed, the membranes can quickly convert from a quiescent, de-induced state to a stimulated rapidly proliferating situation in response to hormonal and/or nutritional signals (11-15). As such, these membranes provide an ideal model system for studying the lipid properties of biological membranes and the regulation of these properties under various physiological conditions. Specifically, in the absence of circulating levels of adrenal glucocorticoids subsequent to bilateral adrenalectomy and following a 24-hr fast, the hepatic SER, and to

a lesser extent, the RER, is decreased in amount and altered in form (i.e., less tubular and fewer bound ribosomes, respectively), and the rate of microsomal membrane synthesis is greatly diminished. Following a single administration of a glucocorticoid (i.e., dexamethasone), a marked proliferation of SER occurs within 2-6 hr following injection of the hormone (11). Moreover, the form of the membranes begins to resemble that of the normal, intact, fasted animal. The effects of glucocorticoid on SER occur concomitant with induced gluconeogenesis and glycogen synthesis and accumulation—all in the absence of additional nutritional factors.

This study is a characterization of the lipid properties of hepatic smooth and rough microsomal membranes obtained from adrenalectomized (ADX) rats and ADX rats treated with glucocorticoids. Significant lipid microviscosity changes were observed using the fluorescent probe DPH and these changes were correlated with the 2 prominent determinants of membrane microviscosity, the cholesterol/phospholipid ratio and the degree of unsaturation of acyl chains.

MATERIALS AND METHODS

Animals

Young adult rats of the Wistar strain (100-150 g) were ADX under ether anesthesia 4-8 days before each experiment. ADX animals were provided with 0.9% saline as drinking water, maintained on a 12-hr light, 12-hr dark cycle and allowed to eat ad libitum. Normal rats which served as controls were housed the same but were provided with tap water. Well-fed ADX rats gain weight, synthesize and store glycogen and maintain plasma insulin and glucose at levels comparable to that of the normal-fed rat (Margolis and Curnow, manuscript in preparation). With fasting, the severe metabolic defects consequent to adrenal insufficiency became evident. Animals were fasted for 20 hr and were divided into the following groups: (a) 36 normal rats, (b) 28 ADX rats which received no further treatment, (c) 30 ADX rats injected intraperitoneally (ip) with pharmacologic doses of dexamethasone (DEX; 2 mg/100 g BW) 2 hr before sacrifice, (d) 30 ADX rats injected ip with DEX 4 hr before sacrifice, and (e) 30 ADX rats injected ip with DEX 6 hr before sacrifice.

Subcellular Fractionation and Preparation of Microsomal Membranes

Animals were sacrificed by decapitation. Their livers were carefully removed to avoid excessive connective tissue contamination, cut into pieces of appropriate size and placed in cold 0.25 M sucrose. The liver specimens were blotted dry, weighed, frozen in either Dry Ice/acetone or liquid nitrogen and stored in vials at -80 C for up to 3 weeks. No qualitative differences were observed when tissue was prepared fresh as opposed to frozen (data not shown). Samples were rapidly homogenized in cold 0.25 M sucrose to make a 20% (wt/vol) homogenate. The homogenate was fractionated by procedures described in a previous publication (11). Briefly, the homogenate was centrifuged twice at 10,000 x g for 20 min, which caused mitochondria, lysosomes and other cellular debris to form a pellet. The postmitochondrial supernatant was collected and brought to 15 mM CsCl by adding sufficient 1 M CsCl. The postmitochondrial supernatant was layered on top of 15 ml 1.3 M sucrose-15 mM CsCl in an SW 27 centrifuge tube and centrifuged at 105,000 x g for 4 hr in a Beckman L5-50 centrifuge (Beckman Instrument Co., Palo Alto, CA). A band, which consisted primarily of smooth microsomes, appeared at the 0.25-1.3 M sucrose interface. A pellet of rough microsomes formed beneath the 1.3 M sucrose. The smooth microsomal band was drawn off, diluted with distilled water, and centrifuged in an SW 41 rotor at 225,000 x g for 1 hr. The rough microsomal pellet was resuspended in 0.25 M sucrose and centrifuged at 225,000 x g for 30 min. Microsomes were subjected to a washing procedure involving resuspension in 0.15 M Tris, pH 8.0, followed by centrifugation and sonication in distilled water that was designed to remove adsorbed proteins, vesicular contents (including very low density lipoprotein [VLDL] particles) and ribosomes. Microsomes were subsequently resuspended in 0.15 M Tris, pH 8.0 and centrifuged once again. Previous determinations of the purity of the microsomal subfractions obtained by these methods using both morphological and biochemical means (11,12) have revealed that contamination by plasma membrane fragments is minimal (i.e., <1% of total protein) (12).

Microviscosity Measurements

Microviscosity was determined according to the Shinitzky and Barenholz method (7) using the lipid-soluble fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene. All measurements were taken at 37 C in a Hitachi Perkin-Elmer spectrofluorimeter Model MPF-3. Microviscosity values ($\overline{\eta}$) are calculated from the Perrin equation $r_o/r = 1 + C(r)\tau/\eta$, where r_o = limiting anisotropy of the probe, taken as 0.362, r = measured anisotropy,

defined as $(I_{\parallel}/I_{\perp} - 1)/(I_{\parallel}/I_{\perp} + 2)$. $I_{\parallel}$ and $I_{\perp}$ are the experimentally determined fluorescence intensities parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. $C(r)$, a function of the molecular shape of the probe, is taken as 8.6×10^5 poise deg^{-1}; T = absolute temperature; τ excited state lifetime, taken as 6 ns at 37 C, from studies with lecithin liposomes (7). Light-scattering was less than 3% and fluorescence values were corrected accordingly. The phospholipid:diphenylhexatriene ratio was always maintained at more than 200:1 (mol/mol) in order to minimize probe-probe interactions or perturbations of the membrane bilayer, or both.

Lipid Analysis

Fatty Acid Analysis. To 0.2 ml of smooth or rough microsomes (0.10 mg protein) was added 50 μgm of pentadecanoic acid in 50 μl chloroform. The lipid was extracted in chloroform/methanol (1:2) by the Bligh and Dyer (18) method. The lipids in the chloroform layer were added to a 5-ml glass ampoule and blown to dryness under nitrogen. The fatty acids were hydrolyzed and methylated using methanolic HCL by Kates' (19) method. Ampoules were flushed with nitrogen and sealed prior to heating at 65 C for 4 hr. The methyl esters of the fatty acids were extracted 3 times with pentane and analyzed by gas chromatography.

Gas liquid chromatography. A Shimadzu GC-FID-c gas chromatograph fitted with a flame ionization detector was used for the quantitative analysis of mixtures of fatty acid methyl esters. Stainless steel columns (3 mm id, 4 mm od) 3 m long were packed with 10% Silar 10 C, 100/120 mesh on Gas Chrom Q support (Applied Sciences Labs, Inc., State College, PA). The column was operated with temperature program from 160-210 C at a rate of 2 C/min with an helium gas flow of 50 ml/min. Peaks were quantitated relative to internal standard, with a Shimadzu Chromtopac E1A integrator.

Cholesterol analysis. Smooth and rough microsomal membranes (0.10 mg) were extracted by the Bligh and Dyer (18) method with 50 μg β-sitosterol added to the membranes as an internal standard prior to extraction. Free cholesterol was quantitated using gas liquid chromatography as already described using 3-m glass columns packed with 3% OV-17, 100-120 mesh, Gas-Chrom Q support (Applied Science Laboratories) at 260 C.

Phospholipid phosphorous. This was extracted by the Folch et al. (16) method and determined by the Bartlett (17) method.

RESULTS

Membrane microviscosity was determined on washed smooth and rough microsomal fractions obtained from fasted, normal, ADX rats and ADX rats treated with DEX. Diphenylhexatriene was added to the membranes and fluorescence depolarization values were calculated from the Perrin equation. The results are presented in Figure 1. The microviscosity value obtained from washed smooth microsomes of normal rats is considerably lower than for the adrenalectomized rats. Following DEX administration to fasted ADX rats, this microviscosity value appears to return to normal after 4 but not 2 hr of treatment. Small changes in microviscosity values are observed in the rough microsomal fraction of liver that are not statistically significant. Rapid proliferation of both protein and lipid components of the smooth, but not the rough, microsomal membranes has been previously reported for ADX animals treated with dexamethasone (11).

Since microviscosity changes are associated with changes in the lipid environment of the probe and since increases in the synthesis of smooth microsomal membrane lipid previously has been observed following DEX administration, it was important to determine the lipid composition of the smooth and rough microsomes obtained from these animals. The results are presented in Table I. Upon adrenalectomy, a decrease in the phospholipid phosphorous is observed in the smooth, but not rough, microsomal fractions as has been previously observed (11). The cholesterol content of the smooth microsomal membrane increases by 60% in ADX rats when compared to normal rats and this results in a 300% increase in the cholesterol/phospholipid ratio. The cholesterol content of the rough microsomes obtained from ADX rats is likewise 60% greater than in normal rats but the cholesterol/phospholipid ratio is increased by 60%, as the phospholipid content of this membrane is unaffected by adrenalectomy.

The lipid composition of the smooth and rough microsomal membranes was further characterized by analyzing the fatty acid composition of the constituent phospholipids present. The results are presented in Table II. Significant shifts in fatty acid composition are observed upon adrenalectomy in smooth microsomal membranes with minor changes in the rough microsomal membranes. A shift to an enhanced long chain polyunsaturated fatty acid composition is observed with a concomitant

decrease in the saturated fatty acids. Arachidonic (20:4) acid content increased significantly whereas that of saturated fatty acids decreased significantly. When the ADX rats are treated with DEX, a return to a fatty acid composition similar to normal animals does not begin until 6 hr of treatment has ensued. A return to fatty acid composition similar to that for normal rats does occur in this time frame and the response to DEX treatment is oberved in both smooth and rough microsomal membranes.

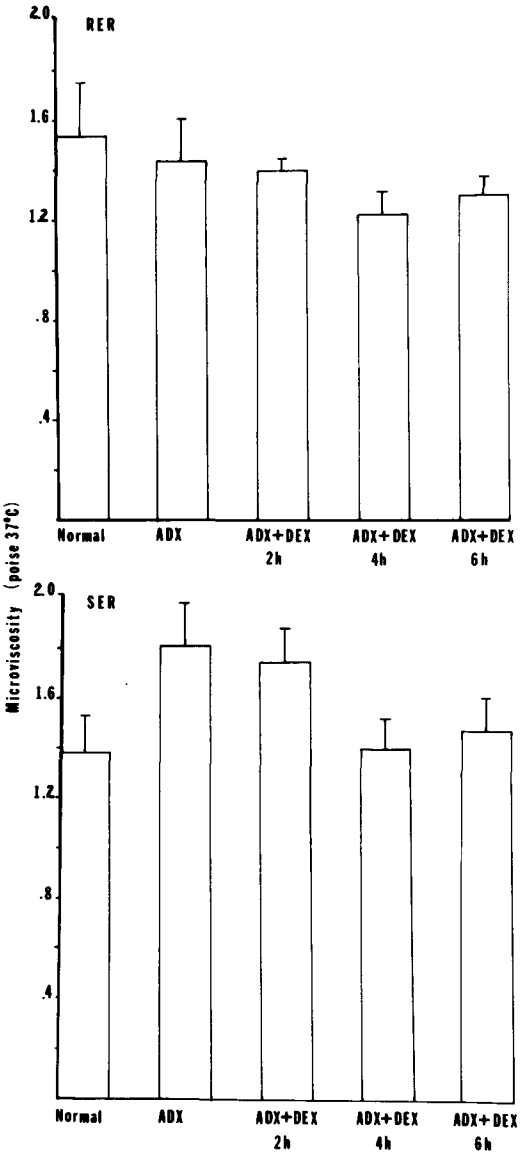

FIG. 1. Each membrane microviscosity value is for duplicate samples determined from 10 to 13 separate animals and are represented as ± SEM. Differences for SER membranes from ADX animals or animals treated with DEX and membranes from control animals are $P > .05$ (Fisher r-test). Differences for RER membrane are not statistically significant.

DISCUSSION

Rapid proliferation of SER was induced in ADX rats by administration of the synthetic glucocorticoid DEX. Although glucocorticoids exert a general catabolic effect on peripheral tissues, the effects on liver are anabolic with acute induction of protein and carbohydrate synthesis using sources of substrates derived from peripheral sources (11,20). Following 2 hr of DEX administration to fasted ADX rats, rapid hepatic microsomal membrane proliferation had begun. After 4 hr of DEX administration, a decrease in smooth microsomal membrane microviscosity was observed, and this microviscosity change continued during the next 2 hr of testing in these experiments. Induction of rapid proliferation in a variety of whole cell preparations apparently is associated with decreased plasma membrane microviscosity and a concomitant decrease in the cholesterol/phospholipid ratio of these membranes (21,22). The results presented here are of interest, since they document that membranes within subcellular compartments undergo a similar decrease in microviscosity and cholesterol/phospholipid ratio when membrane proliferation is induced.

In contrast to the significant changes in cholesterol/phospholipid ratio and microviscosity values observed in smooth microsomal membranes, the rough microsomal membranes remained relatively unchanged in these 2 parameters. Smooth microsomal membranes from ADX rats have a 300% higher ratio of cholesterol/phospholipid and microviscosity values that are 35% higher than for comparable membranes from control animals. In comparison, the cholesterol/phospholipid ratio of rough microsomal membranes from ADX rats increased 60% over control values and there was no statistically significant change in comparable microviscosity values. Our results correlate well with previous ultrastructure studies which document the specific induction of SER proliferation in response to glucocorticoid administration (11). An increase in the cholesterol content of smooth and rough microsomal membrane is observed upon adrenalectomy, and DEX administration returns the membrane cholesterol content to levels found in microsomal membranes obtained from normal rats. It has been observed by Mitro-

TABLE I

Cholesterol and Phospholipid Analysis of Washed Smooth and Rough Microsomal Membranes

	Cholesterol[a] μmol/mg protein	Phospholipid[a] μmol/mg protein	Cholesterol:Phospholipid ratio
SER			
Normal	.173 ± .031 (3)	.61 ± .02 (3)	.28
ADX	.280 ± .045 (4)[b]	.37 ± .01 (4)[b]	.81[b]
ADX + DEX 2 hr	.340 ± .056 (5)[b]	.72 ± .09 (5)	.47[b]
ADX + DEX 4 hr	.169 ± .023 (7)	.80 ± .04 (7)	.21
ADX + DEX 6 hr	.175 ± .018 (7)	.74 ± .07 (7)	.23
RER			
Normal	.091 ± .025 (3)	.57 ± .07 (3)	.16
ADX	.147 ± .032 (4)	.54 ± .02 (4)	.27
ADX + DEX 2 hr	.100 ± .018 (5)	.68 ± .04 (5)	.15
ADX + DEX 4 hr	.077 ± .011 (8)	.83 ± .01 (8)	.09
ADX + DEX 6 hr	.083 ± .010 (7)	.65 ± .03 (7)	.13

[a]All values are mean ± SEM for (N) determinations.

[b]All values are significantly different from normal values. $P > .05$ (Fisher τ test).

poulos and Balasubramanian (3) that, within 3 hr of glucorticoid administration to the ADX rat, both HMG-CoA reductase and cholesterol 7α-hydroxylase activities were returned to values similar to those in normal rats. This is in agreement with our data for the return of the cholesterol content of smooth and rough microsomal membranes 4 hr after DEX injection. Hence, our data on the regulation of hepatic smooth and rough microsomal membrane cholesterol content by DEX is consistent with the work of others on the regulation by glucocorticoids of the 2 major enzymes involved in hepatic cholesterol synthesis and metabolism.

Another important parameter that can influence membrane microviscosity is the fatty acid composition of the membrane phospholipids and this was examined in the smooth and rough microsomal membranes obtained from normal, ADX rats and DEX-treated rats. Fatty acid composition changes were observed in SER

TABLE II

Fatty Acid Content of SER and RER

Fatty acid	Normal	ADX	ADX[a] DEX 2 hr	ADX[a] DEX 4 hr	ADX[a] DEX 6 hr
SER			Mole Percent[a]		
16:0	23	15	17	16	26
16:1	1	1	2	1	1
18:0	22	13	17	16	17
18:1	9	9	13	11	11
18:2	12	17	13	19	17
20:4	27	40	36	32	25
22:6	7	4	3	4	3
Molar S/U[b] ratio	.81	.39	.51	.48	.75
RER			Mole percent[a]		
16:0	23	18	15	16	23
16:1	1	2	1	1	1
18:0	21	22	18	13	21
18:1	8	10	9	11	12
18:2	11	15	14	23	18
20:4	27	30	39	32	23
22:6	9	2	4	4	3
Molar S/U[b] ratio	.80	.68	.50	.41	.77

[a]All values are from duplicate determinations that agree within 10%.

[b]Mol saturated fatty acids/mol unsaturated fatty acids.

and RER membrane preparations and a high concentration of arachidonic acid was observed in these membranes. Changes in fatty acid composition may have a significant effect upon membrane microviscosity parameters. An evaluation has been made of the relative contribution of fatty acid changes compared to other lipid factors such as sterol and phospholipid head group content on microviscosity values observed (9,10). In these studies, changes in fatty acid composition were reported to have only a minor effect on LM cell membrane microviscosity when compared to sterol and phospholipid head group contributions. In our studies, the increased sterol content in smooth and rough microsomal membranes could provide the balance for the arachidonic acid increase and thereby maintain the microviscosity parameter within the limits observed.

DEX administration apparently had no significant effect on the fatty acid composition on the membranes until 6 hr after administration. de Gomez Dumm and coworkers (24) studied the control by glucocorticoids of oxidative desaturation of fatty acids in rat liver and found that glucocorticoids suppress the synthesis of linolenic acid and arachidonic acid by inhibiting the oxidative desaturation of fatty acids in positions 5 and 6. The half-time for the biological effect on these enzymes with dexamethasone was 7 hr. This is in agreement with our observations that changes in membrane fatty acid composition begin by 6 hr after DEX administration. Therefore, we may account for the reduced arachidonic acid content of our membranes after 6 hr by a decrease in the rate of synthesis of arachidonic acid in liver.

Our present studies have focused on the dynamic lipid changes that occur in SER during DEX-induced proliferation. We have demonstrated that cholesterol content of these membranes, when expressed per mg of protein or as per mole phospholipid, changes in a different time frame with regard to glucocorticoid administration than does the fatty acid composition of the constituent phospholipids. Furthermore, the lipid content alterations apparently occur only after the respective biosynthetic enzymes have been induced. In the fatty acid biosynthesis, it would appear that glucocorticoids suppress the synthesis of arachidonic acid perhaps by action upon synthesis of the desaturase enzyme. This results in a regulation of the prostaglandin precursor and may be an important contribution of glucocorticoids to the antiflammatory response of the animal.

The membrane microviscosity changes measured in these experiments correlate well with the lipid compositional changes observed, indicating that the fluidity and composition of the lipid bilayer changes during hormone-induced SER proliferation. What role these lipid changes have in regulation of membrane enzyme constituents has not been determined. Since glucocorticoids are well known to affect protein synthesis at the transcriptional level (20,25), the lipid compositional changes observed in these studies may be entirely accounted for by a change in the level of individual enzymes in the hepatocyte.

It is widely accepted that the action of glucocorticoids is mediated, in part, through inhibition of prostaglandin biosynthesis, but whether this is primarily through direct action upon secretion of prostaglandins (26), through regulation of phospholipase A_2 (27) activity or through inhibition of cyclo-oxygenase activity (28) is still under investigation. Others have suggested that the availability of fatty acid precursors might also be a controlling factor for the biosynthesis of prostaglandins (29). Significant prostaglandin synthesis has been demonstrated recently in rat liver microsomes (30) and the biosynthesis of prostacyclin in rat liver endothelial cells also has been observed (31). We have demonstrated that the arachidonic acid content of liver membrane phospholipid is reduced following glucocorticoid administration. These data suggest that glucocorticoids may act to reduce substrate availability for prostaglandin synthesis in the liver and may contribute significantly to the regulation of prostaglandin production in the liver.

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METHODS

Detection of Hydroxy Fatty Acids in Biological Samples Using Capillary Gas Chromatography in Combination with Positive and Negative Chemical Ionization Mass Spectrometry

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ABSTRACT

The most common method for use in the structural analysis of hydroxy fatty acids in biological samples is the gas chromatography-mass spectrometry (GC-MS) analysis of trimethylsilyl ethers of the methyl esters using electron impact ionization. A comparison of electron impact (EI) with chemical ionization mass spectrometry (CI-MS) shows that CI-MS is the superior technique. All ions necessary for structural analysis are observed at sufficiently high levels of intensity when methane or isobutane are used as reactant gases. The molecular weight can be determined from the ion group M+H, M-15 and M+H-90. The ionic series M+H-n×90 enables one to determine the number of hydroxyl groups. The position of the hydroxyl groups can be derived from the fragments of the α-cleavage of the fatty acid chain. The application of heptafluorobutyrates as derivatives for hydroxy fatty acid methyl esters shows advantages in the trace analysis of these compounds. Heptafluorobutyrates exhibit useful mass fragmentation patterns in the positive as well as in the negative CI mode. With methane as the reactant gas, M+H usually is base peak in positive mass spectra. The ionic series M+H-n×214 leads to the number of hydroxy groups in the molecule. In the negative mass spectra, M and M-20 are indicative for the molecular weight. The ion group m/z 213, 194 and 178 at high levels of intensity is typical for heptafluorobutyrates. The advantage of the application of heptafluorobutyrates is the high sensitivity which can be obtained in trace analysis using negative MS. Heptafluorobutyrates of hydroxy fatty acids gave a 20-fold higher response in the negative scan mode compared to that of the positive. The detection limit for heptafluorobutyrates in negative CI-MS was on the order of 1 fg (10^{-15} g).

INTRODUCTION

Hydroxy fatty acids are formed from unsaturated fatty acids via enzymatic reaction, through autoxidation and during heating of fats in the presence of air (deep frying). Hydroxy fatty acids also are components of cutin in the cuticle of plants. They are characteristic substances in the cellular fatty acid composition of bacteria. Hydroxy fatty acids can be used to elucidate the structure of unsaturated fatty acids by specific oxidation to locate the position of the double bonds.

The most common method for use in the structural analysis of these substances is the gas chromatography-mass spectrometry (GC-MS) analysis of trimethylsilyl ethers of the methyl esters using electron impact (EI) ionization (1-3). In this paper, we hope to demonstrate the superiority of positive and negative chemical ionization (CI) over EI ionization mass spectrometry for structural and trace analysis.

EXPERIMENTAL

Methods

Hydroxy fatty acids of definite structure can be produced by enzymatic oxidation with various lipoxygenase preparations (3-5), by chemical oxidation of unsaturated fatty acids with $KMnO_4$ or OsO_4 (6,7) and from fat extracts of bacteria (8,9). The hydroxy fatty acids presented in this study were obtained by the following methods or commercial suppliers: 2-hydroxy octadecanoic acid (Supelco, Bellefonte, PA), 9-hydroxy octadecanoic acid (4), 12-hydroxy methyl octadecanoate (Serva, Heidelberg, W. Germany), methyl ricinoleate (Supelco, Bellefonte, PA), 9,10-dihydroxy octadecanoic acid (6), 12,13-dihydroxy octadecanoic acid (4), 9,10,12-trihydroxy octadecanoic acid (6), 9,10,12,13-tetrahydroxy octadecanoic acid (6), and bacterial acid methyl ester mixture (Supelco, Bellefonte, PA).

All self-prepared substances were purified by

recrystallization and thin layer chromatography (TLC) and characterized by chromatographic parameters. The identity of the structures was confirmed by established GC-MS procedures of the trimethylsilyl ethers with EI ionization (1-3). Hydroxy fatty acids were methylated with diazomethane (10). Trimethylsilyl ethers were made by addition of 0.3 ml N,O-bis-(trimethylsilyl)-acetamide with 10% trimethylchlorosilane (BSA/10% TMCS) to the sample at a temperature of 70 C and a reaction time of 1 hr in a closed reaction vessel. Heptafluorobutyrates were formed by dissolving the sample in 0.1 ml benzene and adding 0.1 ml heptafluorobutyric anhydride. This mixture was also held at 70 C for 1 hr in a closed reaction vessel. After conversion to the trimethylsilyl or heptafluorobutyryl derivatives, excess reagents were removed under a gentle stream of nitrogen. These derivatives of hydroxy fatty acid methyl esters were separated by GC using a glass capillary SE 54 of 30 m length and 0.3 mm id (Jaeggi, Trogen, Switzerland); carrier gas: helium at 2.5 ml/min; temperature program: 1 min at 90 C, increased at 10 C/min (or 20 C/min) up to 250 C, then held at 250 C isothermally. Splitless injection was applied according to Grob and Grob (11) into the column at 90 C. The gas chromatographic eluate was introduced directly into the ion source of a quadrupole mass spectrometer through an all-glass, open-split interface (12). The interface was held at 250 C. The combined ion source for EI ionization and CI was operated at an energy level of 70 eV and an emission current of 300 μA at a temperature of 200 C. For positive and negative CI, methane and isobutane were preferred as reactant gases at an ion source pressure of ca. 0.2 Torr. Mass spectral data were acquired using a cyclic scan procedure with a scan time of 1.9 sec and a holding time of 0.1 sec at the low mass. The mass range used was usually between m/z 70 and m/z 700. For the recording of heptafluorobutyrates of polyhydroxy fatty acid methyl esters, the scan was extended to m/z 990. Both positive and negative CI mass spectra were alternately acquired in a cyclic scan with a scan time of 1.2 sec for the positive as well as for the negative record.

Reagents

The reagents used were: linoleic acid (Roth, Karlsruhe, W. Germany); bacterial acid methyl ester mixture, 2-hydroxy octadecanoic acid, methyl ricinoleate, (Supelco, Bellefonte, PA); 12-hydroxy methyl octadecanoate, heptafluorobutyric anhydride and silylation reagents BSA and TMCS (Serva, Heidelberg, W. Germany); all the other chemicals were pro analysi (Merck, Darmstadt, W. Germany).

GC-MS System

A Gas Chromatography-Mass Spectrometry System 4000 with Incos Data System (Finnigan, Munich, W. Germany) was used.

RESULTS AND DISCUSSION

In this comparative study on the evaluation of various ionization methods for the structure elucidation of hydroxy fatty acids by GC-MS, we investigated hydroxy fatty acids of different origin. The hydroxy fatty acids were produced by various lipoxygenase preparations (3,5), with fruit homogenates (4), by chemical oxidation (6,7), from plant cuticle (13), from fat extracts of bacteria (8,9) and deep frying fats (14,15). The hydroxy fatty acids included saturated and unsaturated compounds of different chain lengths. For clarity, we present in this paper only the results obtained from saturated octadecanoic acids with different numbers of hydroxyl groups. The fragmentation mechanisms found with this group of compounds, however, are representative of the varieties of all investigated substances.

In Figure 1, the mass spectra of the trimethylsilyl ether of 9,10-dihydroxy methyl

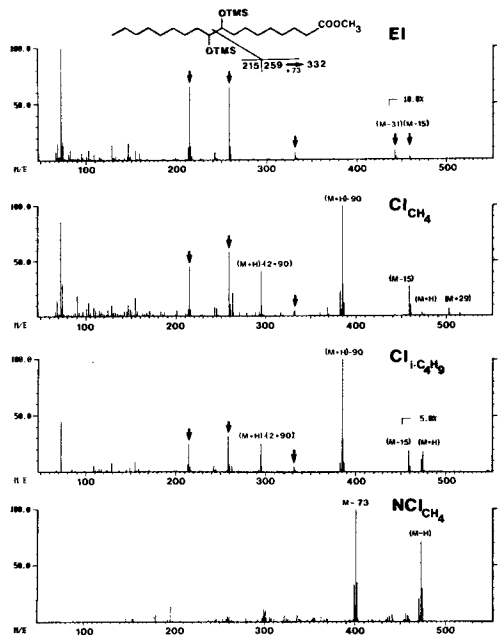


FIG. 1. Mass spectra of methyl 9,10-dihydroxyoctadecanoate as trimethylsilyl ether using various ionization modes.

stearate obtained under different ionization conditions are shown. The upper 3 spectra are records of positive ions whereas the spectrum below shows the negative ions. With EI ionization, ions in the molecular weight (MW) region are of very weak intensity; fragments resulting from the α-cleavage at the hydroxyl groups are observed at high levels of intensity. These fragments are indicative of the position of the hydroxyl groups in the fatty acid chain (1-3). CI with methane or isobutane as reactant gases yields the same fragments and additionally intense ions of M+H-90 and of M+H-2x90. These fragments result from the elimination of trimethylsilanol from the hydroxy fatty acid. This ion series M+H-nx90 yields the number (n) of hydroxyl groups in the fatty acid (16) and can be used to confirm the MW. In the CI mass spectra of all investigated hydroxy fatty acids, these ions were found at sufficiently high levels of intensity. With isobutane, the group of M+H, M-H and M-15 is indicative of the MW; with methane as the reactant gas, M+29 and M+41 also were found. The negative CI mass spectrum with methane yields only 2 intense ions, i.e., M-H and M-73, resulting from the abstraction of the trimethylsilyl group. These 2 ions only confirm the MW and the presence of a trimethylsilyl ether. Figure 2 shows the CI mass spectra of a series of octadecanoic acids with an increasing number of hydroxyl groups derivatized as trimethylsilyl ethers. With methane as reactant gas in all 4 examples, the ions in the MW region are of medium intensity, sufficient to determine the MW as described before. The series of fragments resulting from the elimination of trimethylsilanols can be found easily in the spectra, indicating the number of hydroxyl groups in the molecule. It is obvious that with an increasing number of hydroxyl groups in the molecule the fragmentation pattern becomes more complex, but the knowledge of the MW, together with the retention time on a suitable gas chromatographic column, helps to confirm the chain length and the number of hydroxyl groups. The fragments resulting from the α-cleavage, which are indicative of the position of the hydroxyl groups in the chain, are observed at high levels of intensity. These fragments are shown in the fragmentation schemes and are marked by arrows in the spectra.

As an example for the application of the method in practice, the GC-MS run of the polar fraction of a processed deep frying fat after transesterification and trimethylsilylation is shown in Figure 3. This fraction was isolated according to Guhr and Waibel (15), proposed as an IUPAC method designed to assess the

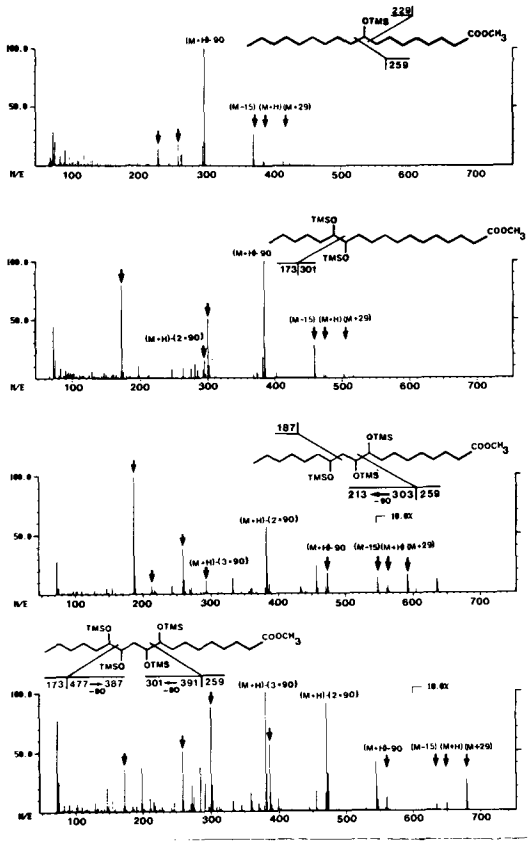


FIG. 2. Mass spectra of the trimethylsilyl ethers of methyl hydroxy octadecanoates using positive CI with methane.

acceptability of a deep frying fat. In addition to the standard procedure, the polar fraction consisting of dimeric and polymeric triglycerides was hydrolyzed, methylated and trimethylsilylated. The GC-MS run shown was performed using the cyclic scan method in the mass range from m/z 70 to 700 as described and CI with methane. In the upper line of Figure 3, the total ion current (TIC) is shown in which large amounts of methyl palmitate, oleate and stearate dominate (peaks a,b and c). The 2 hydroxylated fatty acids d and e are present in the sample in very low concentrations. With the mass fragmentograms of the M+H ions, reconstructed from the data of the cyclic scan, methyl palmitate (m/z 271), methyl oleate (m/z 297) and methyl stearate (m/z 299) can be identified easily. The confirmation of these findings can be achieved by looking for additional typical ions as M-H and M+29 for each substance. Two examples of this type are found in the mass fragmentograms shown, namely m/z 297 corresponds to M-H of methyl stearate and

m/z 299 corresponds to M+29 of methyl palmitate. This example shows that GC-MS with CI is a very useful method to detect fatty acid methyl esters in biological samples.

Peak d consists of a mixture of 9- and 10-hydroxy methyl stearate all as trimethylsilyl ethers. The trimethylsilyl ethers of hydroxy fatty acid methyl esters exhibit a fragmentation pattern with intense ions of M+H-90 which are for all monohydroxy stearates m/z 297 and for dihydroxy stearates m/z 385 (peak e). The peak of m/z 385 in the mass fragmentogram at the retention time of the monohydroxy stearates is the M-H of these compounds.

The occurrence of the same characteristic ions in various fatty acids and hydroxy fatty acids arises from the fragmentation mechanisms explained, i.e., the M+H-90 ion of a saturated hydroxy fatty acid has the same m/z value as the M+H ion of the monounsaturated fatty acid of the same chain length. This correspondence does not lead to misinterpretations because of the different retention times of the 2 compounds in GC (see m/z 297 in Fig. 3).

The position of the hydroxyl groups can be determined easily by means of the fragments resulting from the α-cleavage (16). In the lower part of Figure 3, mass fragmentograms of the characteristic ions of trimethylsilyl ethers of 9- and 10-hydroxy methyl octadecanoates were recorded. Peak d consists of a mixture of 9- and 10-hydroxy methyl octadecanoate because the typical ion pairs for the 9-position m/z 259 and 229, as well as for the 10-position m/z 273 and 215, are present.

Peak e consists of 9,10-dihydroxy methyl octadecanoate, a finding deduced from the ion pair m/z 259 and 215. The complete mass spectrum of this compound was nearly identical to the mass spectrum prepared from a test substance shown in Figure 1. Furthermore, small amounts of a mixture of unsaturated monohydroxy methyl octadecanoates can be observed in Figure 3 with shorter retention times as the saturated monohydroxy compounds. This is in accord with test substances determined on the capillary column used. Without discussing the interpretation in detail, 12-hydroxy methyl octadecenoate was found to be the main component with the ion pair m/z 299 and 187 whereas the ions recorded at m/z 259 and 273 in Figure 3 derived from minor components of the mixture.

Negative CI-MS has been introduced recently as a very sensitive method for the detection of trace amounts of drug and pesticide residues in biological samples. We studied the applicability of this method for the analysis of hydroxy fatty acids.

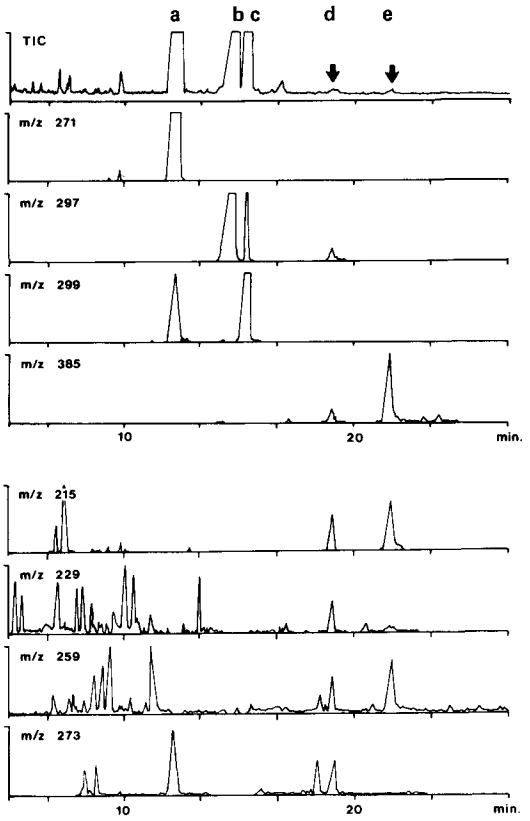


FIG. 3. GC-MS run of the polar fraction of a processed deep frying fat after methylation and trimethylsilylation applying chemical ionization with methane; (a) methyl palmitate; (b) methyl oleate; (c) methyl stearate; (d) methyl 9-hydroxy and 10-hydroxy octadecanoate as trimethylsilyl ether; (e) methyl 9,10-dihydroxy octadecanoate as trimethylsilyl ether.

Trimethylsilyl ethers are not very well suited for negative CI-MS because of their lower sensitivity compared to the positive CI. This is a disadvantage in trace analysis. For this purpose, heptafluorobutyrates were found to be better derivatives. This can be seen from Figure 4, where a mixture of fatty acids and hydroxy fatty acids was analyzed as methyl esters after derivatization with heptafluorobutyric anhydride. The GC-MS run was recorded alternating in the positive and negative CI mode. In the positive CI mode, all fatty acids and hydroxy fatty acids were recorded with the TIC, whereas in the negative CI mode, only the heptafluorobutyrates of the hydroxy fatty acids in the mixture were observed in the TIC record. In the sample shown, the heptafluorobutyrates gave a 20-fold higher response in the negative TIC compared to that of the positive.

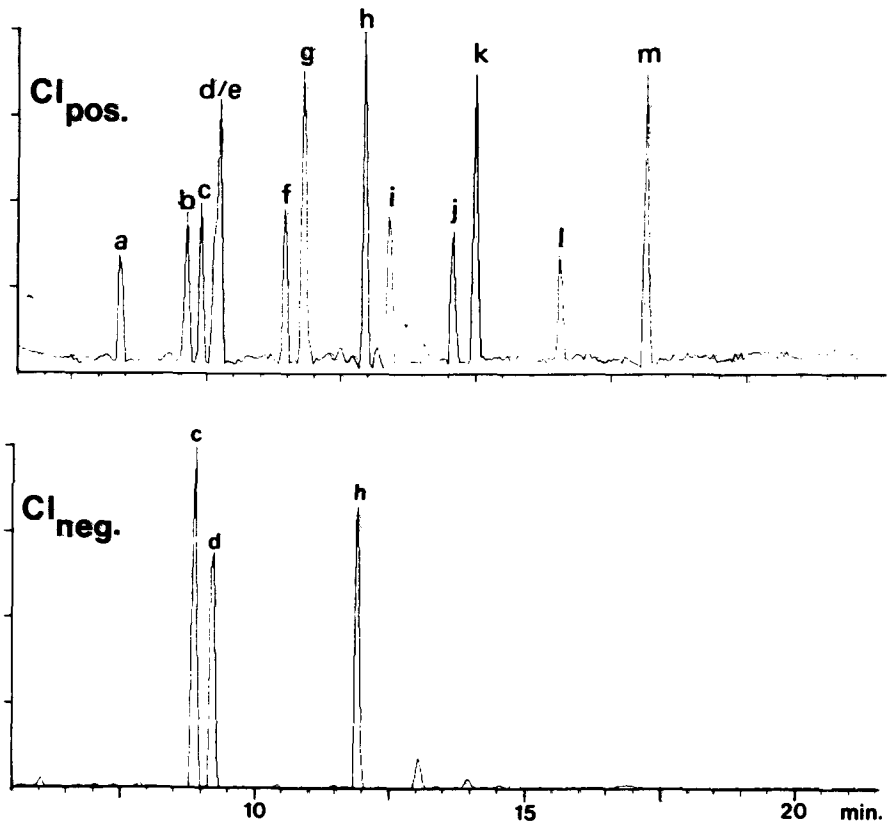
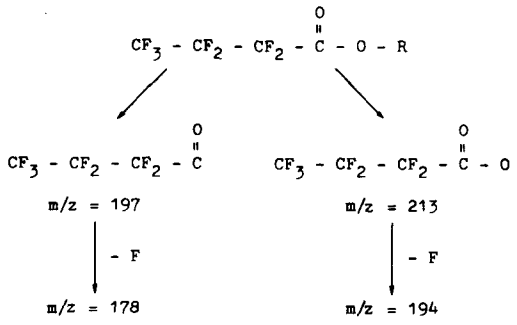


FIG. 4. GC-MS run of a derivatized bacterial acid mixture including hydroxy fatty acids as heptafluorobutyrates using positive and negative CI with methane; (a) methyl myristate; (b) methyl 12-methyl tetradecanoate; (c) methyl 2-hydroxy tetradecanoate as heptafluorobutyrate; (d) methyl 3-hydroxy tetradecanoate as heptafluorobutyrate; (e) methyl pentadecanoate; (f) methyl palmitoleate; (g) methyl palmitate; (h) methyl 2-hydroxy hexadecanoate as heptafluorobutyrate; (i) methyl heptadecanoate; (j) methyl oleate; (k) methyl stearate; (l) methyl nonadecanoate; (m) methyl arachidate.

The positive CI mass spectra of a series of octadecanoic acid methyl esters with increasing numbers of hydroxyl groups as heptafluorobutyrates are shown in Figure 5. All spectra exhibit the pseudo molecular ion (M+H) as base peaks accompanied by smaller M+29 and M+41 ions. For the tetrahydroxy compound, however, the pseudo molecular ion could not be recorded because the m/z was outside of the mass range of our instrument. The mass spectra exhibit only a few intense ions and are, therefore, easily interpreted. In analogy to the mass fragmentation pattern of trimethylsilyl ethers, a successive elimination of all heptafluorobutyric acids from the molecule corresponds to the M+H of an unsaturated fatty acid methyl ester with the same number of double bonds, as hydroxyl groups have been present in the original compound.

The negative CI mass spectra of the heptafluorobutyrates of the hydroxy fatty acids (Fig. 6) show a typical group of intense ions at m/z 178, 194 and 213. This group is frequently accompanied by the ions m/z 197 and 232 (213+F). The formation of these ions is demonstrated in the following fragmentation scheme:



Additionally, we observed, with increasing numbers of heptafluorobutyrate groups in the

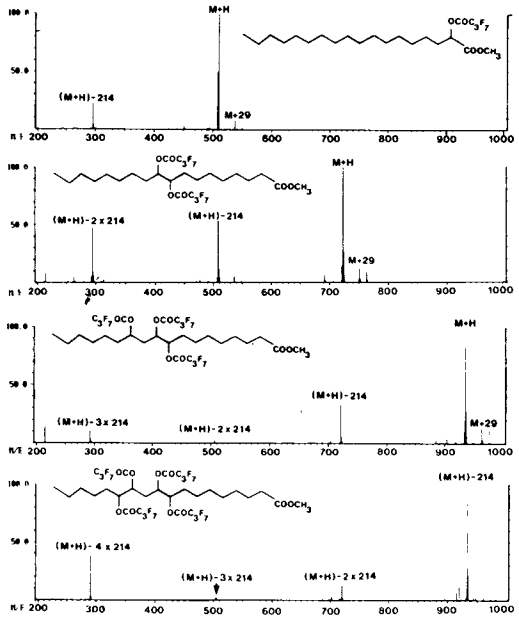


FIG. 5. Mass spectra of the heptafluorobutyrates of the methyl hydroxy octadecanoates using positive CI with methane.

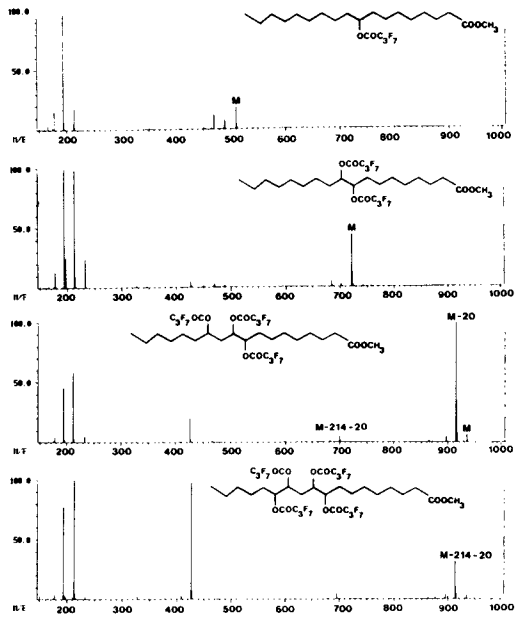


FIG. 6. Mass spectra of the heptafluorobutyrates of the methyl hydroxy octadecanoates using negative CI with methane.

molecule, the appearance of the ion m/z 427, which may result from a protonated dimer of the heptafluorobutyrate (m/z 213). Information about the individual hydroxy fatty acid exists only in the ions M, M-20 and M-2x20 which are indicative of the MW. M-20 and M-2x20 result from the elimination of hydrogen fluoride from the molecule. Again, the M or M-20 for the tetrahydroxyoctadecanoic acid could not be detected because of the mentioned instrumental limitations, but in this particular case, a clear M-20-214 and a weaker M-214 ion resulting from the elimination of heptafluorobutyric acid could be observed (Fig. 6). The same type of fragments could be found in the other spectra only in minute intensities. The comparison of the fragmentation patterns shown in Figures 5 and 6 demonstrates the different mechanisms for the formation of positive and negative ions during the CI process. In positive CI, the ions of the series M+H-nx214 are seen because the positive charge is thought to be at the methyl ester group as observed with fatty acids; in negative CI, the heptafluorobutyrate group captures the thermal electrons and, after bond cleavage, the negative charge remains at the heptafluorobutyrate resulting in the series m/z 178, 194 and 213, as shown in the fragmentation scheme. The value of negative CI-MS for practical application in trace analysis may not be recognized from the examples described. Therefore, the utility of negative CI-MS can be demonstrated with heptafluorobutyrates of hydroxy fatty acids in the polar fraction of a processed deep frying fat. With positive CI as well as EI ionization, it has been difficult to identify unequivocally the occurrence of a monounsaturated and a saturated monohydroxy octadecanoic acid in our chromatographic system. Both compounds were partially separated as trimethylsilyl ethers as well as heptafluorobutyrates. But, in the positive ion record, the appearance of M+H and M-H together with the isotope peaks from both compounds make it difficult to relate the ions to the 2 substances. With negative CI, it is easy to relate the peaks to the corresponding substances using M and the series M-nx20. In this particular case shown in Figure 7, the use of the M-2x20 ions was the most suitable.

Finally, we want to report on another application in practice, which we used to detect hydroxy fatty acids in the lipids extracted from pork liver. After transesterification and derivatization, trimethylsilyl ethers and heptafluorobutyrates were analyzed. Various unsaturated monohydroxy and saturated dihydroxy octadecanoic acids could be detected (unpublished results). In order to solve this problem, we used 12-hydroxy methyl octadecanoate, 12-hydroxy methyl octadec-9-enoate and 9,10-dihydroxy

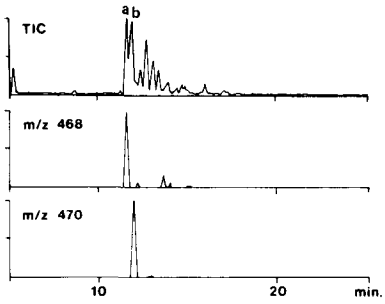


FIG. 7. GC-MS run in the region of methyl hydroxy octadecanoates of the polar fraction of a processed deep frying fat after methylation and heptafluorobutyration applying negative chemical ionization with methane; (a) methyl 9-hydroxy octadecenoate as heptafluorobutyrate; (b) methyl 9-hydroxy octadecanoate as heptafluorobutyrate.

methyl octadecanoate to find out the detection limit of the hydroxy compounds found in pork liver. Using the trimethylsilyl ethers as well as the heptafluorobutyrates, we found the minimum amount necessary to obtain a complete mass spectrum is on the order of 10 ng in positive CI-MS. With multiple ion detection, 10 pg of hydroxy fatty acids could be detected selecting the M+H-90 ions for trimethylsilyl ethers or M+H-214 ions for heptafluorobutyrates.

In negative CI-MS, 1 fg of heptafluorobutyrates was detectable using the ions m/z 178 and 194 for mass fragmentography. It has to be noticed that the negative CI-MS method does not yield any information about the structure of the detected substances but only the confirmation of the presence of a heptafluorobutyrate. In this case, the mass spectrometer is to be considered a very sensitive and specific gas chromatographic detector for heptafluorobutyrates. Further information about the identity of the analyzed substances must be drawn from gas chromatographic data using test substances and capillary columns of different polarity.

Similar investigations with capillary GC and electron capture detection resulted in a detection limit of ca. 10 pg heptafluorobutyrates of hydroxy fatty acids (17). Thus, comparing the detection limits of the 2 methods, negative CI-MS was found to be 3 or 4 orders of magnitude more sensitive than electron capture detection with heptafluorobutyrates.

In conclusion, we suggest positive CI-MS of trimethylsilyl ethers as a method for structure analysis and negative CI-MS of heptafluorobutyrates for trace analysis of hydroxy fatty acids.

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The Preparation and Characterization of the Methoxy Derivatives of Polyunsaturated Fatty Acids: Stabilized Product Analogs of Lipoxygenase Catalysis

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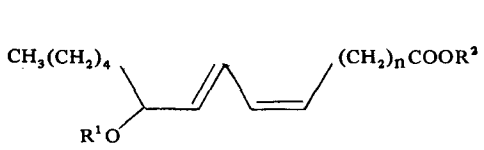
ABSTRACT

The products from the action of soybean lipoxygenase on a series of polyunsaturated fatty acids have been converted in 2 steps into the corresponding methoxy derivatives. The product hydroperoxides were reduced in situ to alcohols with sodium borohydride. The ethers were generated by treatment of the alcohols with sodium hydride and methyl iodide in tetrahydrofuran. 13-Methoxy-9(Z),11(E)-octadecadienoic, 15-methoxy-11(Z),13(E)-eicosadienoic and 15-methoxy-5(Z),8(Z),11(Z), 13(E)-eicosatetraenoic acids were thus prepared. The methyl ethers were analyzed and the structures established by high performance liquid chromatography, nuclear magnetic resonance and infrared spectrometry, polarimetry and mass spectroscopy (as methyl esters). The methylation reaction proceeds without molecular rearrangements or racemiziation.

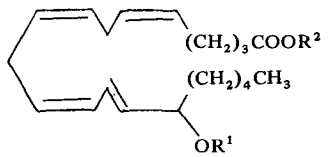
INTRODUCTION

Lipoxygenase is widespread in nature and well known, as it was first isolated from soybeans in crystalline form in 1947. In recent years, mammalian forms of the enzyme with important physiological roles have been observed. Specific modifiers of lipoxygenase and associated peroxidase activities are relatively scarce. One obvious feature of lipoxygenase chemistry is the inherent instability of the product hydroperoxides. In order to provide novel compounds suitable for the study of the enzymatic processes, we have prepared a series of stabilized derivatives of the products of lipoxygenase catalysis. This has been done by the straightforward application of the Williamson ether synthesis to the preparation of the appropriate methoxy compounds from the corresponding enzymatically formed alcohols. These derivatives would not be capable of further metabolism in a fashion similar to the actual products, but by virtue of their close structural similarity, may influence one or more of the enzymes involved. This report details our findings on the conversion of linoleic, 11,14-eicosadienoic and arachidonic acids into methoxy derivatives (**2**,**5** and **8**) using soybean lipoxygenase to provide the precursor alcohols (**1**,**4** and **7** Scheme I).

The etherification of fatty alcohol **1** in methanolic acid has been reported (1) and the resulting derivatives have been used in structural analyses (2). This approach to the synthesis of methoxy derivatives was invariably accompanied by isomerization (and presumably racemization) through the formation of carbonium ion intermediates. Typical etherification reactions in lipid chemistry (i.e., the preparation of glyceryl ethers) involve conversion of the fatty alcohol into the corresponding halide or sulfonate, followed by displacement with alkoxide (3,4). Since stereochemistry is not maintained in these experiments and application to allylic alcohols would be expected to result in some degree of isomerization, we have used the fatty acid as the nucleophilic component in order to preserve its structure and chirality.



1 n = 7, R^1 = H, R^2 = H
2 n = 7, R^1 = CH_3, R^2 = H
3 n = 7, R^1 = CH_3, R^2 = CH_3
4 n = 9, R^1 = H, R^2 = H
5 n = 9, R^1 = CH_3, R^2 = H
6 n = 9, R^1 = CH_3, R^2 = CH_3



7 R^1 = H, R^2 = H
8 R^1 = CH_3, R^2 = H
9 R^1 = CH_3, R^2 = CH_3

SCHEME I

MATERIALS AND METHODS

Fatty acids were obtained in greater than 99% purity from Nu-Chek-Prep (Elysian, MN). The enzyme was Type I lipoxygenase from soybeans (Sigma Chemical Co., St. Louis, MO). Preparative scale purifications were carried out by chromatography on silica gel using the Still et al. method (5). The solvent systems used were as follows: alcohols, acetic acid/acetone/hexane (1:25:74); ethers, acetic acid/acetone/hexane (1:15:84). The preparation of the alcohols in general followed established procedures (6,7). The methyl esters were prepared by the method of Stoochnoff and Bènoiton (8). Methyl esters were prepared by treating the acids with a 5-fold excess of CH_2N_2 in diethyl ether for 5 hr at 4 C. The solvent was removed in a stream of nitrogen and the esters were purified by high performance liquid chromatography (HPLC).

13-Hydroxy-9(Z),11(E)-octadecadienoic acid (**1**): ^{1}H NMR δ 6.46 (dd,1H), 5.94 (t,1H), 5.69 (m,1H), 5.36 (m,1H), 4.16 (q,1H), 2.21 (m,4H), 1.31 (m,18H), 0.89 (bt,3H). ^{13}C NMR ppm 179.54, 135.84, 132.89, 128.04, 125.98, 73.08, 37.38, 34.16, 31.91, 29.55, 29.04 (3), 27.77, 25.22, 24.79, 22.73, 14.17. IR cm^{-1} 3327, 2922, 2857, 2667, 1710, 1462, 1409, 1247, 1127, 1083, 985, 949, 837, 735.

15-Hydroxy-11(Z),13(E)-eicosadienoic acid (**4**): ^{1}H NMR δ 6.51 (dd,1H), 5.97 (t,1H), 5.74 (m,1H), 5.45 (m,1H), 4.17 (q,1H), 2.24 (m,4H), 1.29 (m,22H), 0.89 (bt,3H). ^{13}C NMR ppm 179.56, 135.74, 132.95, 127.93, 126.01, 73.08, 37.38, 34.21, 31.91, 29.72, 29.45 (2), 29.31 (2), 29.15, 27.87, 25.19, 24.84, 22.73, 14.14.

15-Hydroxy-5(Z),8(Z),11(Z),13(E)-eicosatetraenoic acid (**7**): ^{1}H NMR δ 6.56 (dd,1H), 5.99 (t,1H), 5.78 (m,1H), 5.45 (m,1H), 5.39 (t,4H), 4.22 (q,1H), 2.90 (m,4H), 2.36 (t,2H), 2.10 (t,2H), 1.73-1.29 (m,10H), 0.89 (bt,3H). ^{13}C NMR ppm 178.70, 136.25, 130.07, 128.94 (2), 128.61, 128.10, 127.61, 125.39, 72.79, 37.22, 33.37, 31.80, 26.52, 26.17, 25.71, 25.08, 24.57, 22.62, 14.06.

13-Methoxy-9(Z),11(E)-octadecadienoic acid (**2**): ^{1}H NMR δ 6.44 (dd,1H), 5.99 (t,1H), 5.54 (m,1H), 5.36 (m,1H), 3.55 (q,1H), 3.27 (s,3H), 2.27 (m,4H), 1.32 (m,18H), 0.88 (bt,3H). ^{13}C NMR ppm 179.78, 133.76, 132.57, 127.99, 127.91, 82.62, 56.10, 35.68, 34.19, 31.97, 29.64, 29.12 (3), 27.79, 25.17, 24.79, 22.70, 14.14. IR cm^{-1} 2919, 2852, 2669, 1711, 1469, 1413, 1287, 1244, 1189, 1097, 988, 952, 737.

15-Methoxy-11(Z),13(E)-eicosadienoic acid (**5**). ^{1}H NMR δ 6.44 (dd,1H), 5.99 (t,1H), 5.54 (m,1H), 5.35 (m,1H), 3.58 (q,1H), 3.27 (s,3H), 2.24 (m,4H), 1.29 (m,22H), 0.88 (bt,3H). ^{13}C NMR ppm 179.67, 133.87, 132.81, 127.99 (2), 82.64, 56.26, 35.81, 34.16, 32.05, 29.77, 29.55 (2), 29.39 (2), 29.23, 27.93, 25.25, 24.87, 22.78, 14.23.

15-Methoxy-5(Z),8(Z),11(Z),13(E)-eicosatetraenoic acid (**8**). ^{1}H NMR δ 6.49 (dd,1H), 6.02 (t,1H), 5.60 (m,1H), 5.39 (t,5H), 3.61 (q,1H), 3.28 (s,3H), 2.90 (m,4H), 2.36 (t,2H), 2.10 (t,2H), 1.74-1.27 (m,10H), 0.88 (bt,3H). ^{13}C NMR ppm 178.80, 134.52, 130.74, 129.04 (2), 128.72, 128.21, 127.77, 127.39, 82.48, 56.24, 35.73, 33.48, 31.99, 26.66, 26.28, 25.82, 25.19, 24.70, 22.73, 14.17.

RESULTS AND DISCUSSION

In situ reduction of the lipoxygenase reaction (pH = 9.2) with sodium borohydride, followed by acidification and extraction, provided the desired alcohols in good yields after purification by adsorption chromatography (Table I). The specificity of the enzyme, known to be ω6 (9), provided single isomers of the hydroperoxides in each case. This was confirmed by HPLC analysis of the alcohols. Half-gram quantities of the fatty acids thus could be processed to the corresponding ω6 alcohols. Selective methylation of the alcohol was achieved in each case by treatment with sodium hydride and methyl iodide in tetrahydrofuran at room temperature. The products were isolated in reasonable yields (Table I) by acidification and extraction, followed by purification on adsorption chromatography. Again, HPLC analysis demonstrated the absence of any isomeric components.

The ^{1}H NMR spectrum for each ether

TABLE I

Step-by-Step Synthetic Yield Summary (%)

Fatty acid	ω6-Alcohol	ω6-Methyl ether
18:2	90	78
20:2	74	81
20:4	60	40

displayed a new singlet absorbance due to the methoxy group (3.26 ppm) and a concomitant shift in the signal (0.58 ppm upfield) due to the proton at the position of oxygen substitution relative to the alcohol. In the proton-decoupled ^{13}C NMR spectrum, the signal at ∿73 ppm in each alcohol was replaced by a signal at ∿82 ppm in the corresponding ether. In each ether spectrum, an absorbance at ∿56 ppm due to the methoxy carbon also was observed. The threat of bond migration or rearrangement in the sensitive double bond regions of these compounds posed by the strenuously basic reaction conditions was considered from the outset. The olefinic regions of the proton spectra were particularly informative in this regard. At 90 mHz, the region was nearly resolved into a set of 4, one-proton multiplets for the *cis,trans*-dienol acids as has been previously reported (20). Only the positions of the signal due to the olefinic proton vicinal to the oxygenated carbon (revealed by homonuclear decoupling) were changed (∿0.2 ppm shift, upfield) when the ether spectra were compared with those of the corresponding alcohols. In the tetraenoic acid, these resonances were joined by a 4-proton signal with characteristic *cis*-olefinic appearance. Again, the only change in this region of the spectrum upon conversion from alcohol to methyl ether was the small upfield shift for the signal due to the proton nearest the carbon bearing the oxygen atom. These observations indicated that the double bond system was not rearranged by treatment with base. Nonrearrangement in the olefinic region was also confirmed by infrared spectroscopy. For example, compounds **1** and **2** displayed virtually identical spectra in the region characteristic of olefinic CH out-of-plane bending deformations below 1000 cm^{-1}. Distinctive bands at 985 and 950 cm^{-1} (*cis,trans*-conjugated diene) (11) were present in both spectra. The prospect of racemization during methylation also was investigated in this system (**1→2**) by measuring optical rotations for the starting alcohol and ether product (Table II). The methoxy derivative rotates polarized light to approximately the same extent as does the alcohol but in the opposite direction. While this data does not establish the optical purity of the ether, complete racemization is clearly ruled out. The Williamson ether synthesis is known to be stereospecific in saturated systems (12), but this apparently is the first case in which the

TABLE II

Specific Optical Rotations: $[\alpha]_{\lambda}^{30}$ (C10.0, Ethanol)

λ	1	2
589	+1.60	-2.00
578	+1.68	-2.09
546	+1.98	-2.37
436	+3.82	-3.93
405	+4.86	-4.61

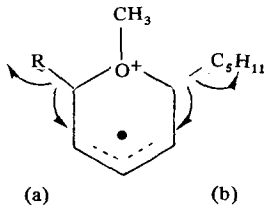
TABLE III

Major Fragments in the Mass Spectroscopic Analysis

m/e	Relative abundance	Assignment
	3	
167	22.7	M- $\cdot(CH_2)_7COOCH_3$
253	100.0	M- $\cdot C_5H_{11}$
292	15.9	M- $HOCH_3$
293	8.3	M- $\cdot OCH_3$
324	94.4	M
	6	
167	26.9	M- $\cdot(CH_2)_9COOCH_3$
281	100.0	M- $\cdot C_5H_{11}$
320	21.6	M- $HOCH_3$
321	10.1	M- $\cdot OCH_3$
352	87.6	M
	9	
167	100.0	M- $\cdot CH_2CH{=}CHCH_2$-$CH{=}CH(CH_2)_3COOCH_3$
277	25.4	M- $\cdot C_5H_{11}$
316	52.7	M- $HOCH_3$
317	16.8	M- $\cdot OCH_3$
348	6.6	M

stereochemistry of an allylic alcohol has been monitored.

Mass fragmentation patterns for the methyl ethers (as their methyl esters; **3,6** and **9**) were obtained at 9 eV (Table III). In addition to abundant molecular ions, these derivatives showed a strong tendency to fragment at the ends of the double bond system. We suggest a mechanism involving a cyclic intermediate to account for this pattern.



SCHEME II

As expected for compounds containing both methyl ether and methyl ester groups, fragments due to loss of methanol and methoxy radical also were present. It is evident from the foregoing that allylic alcohols in polyunsaturated fatty acids can be selectively methylated without racemization at the substituted position or bond migration in olefinic regions elsewhere in the molecule. This straightforward synthesis provides access to a new class of fatty acids structurally related to the products of lipoxygenase catalysis.

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Gangliosides from Rat Cerebellum: Demonstration of Considerable Heterogeneity Using a New Solvent for Thin Layer Chromatography

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ABSTRACT

Using a new solvent (methyl acetate/*n*-propanol/chloroform/methanol/0.25% aqueous KCl, 25:20:20:20:17, v/v) and high performance silica gel thin layer chromatographic plates, all common gangliosides found in brain can be easily separated with 1 hr. This system is reproducible and "tailing" is negligible compared with previous solvents. When such a system is applied to separate the gangliosides found during the development of the rat cerebellum, a considerable heterogeneity is observed. Data are presented (using rechromatography experiments, fractionation on DEAE-Sephadex, treatment with neuraminidase or alkaline medium and carbohydrate analysis) suggesting that the complex profiles obtained with this chromatographic system are not due to chromatographic artifacts but result from the high resolving power of the system. After separation by ion-exchange chromatography, 28 gangliosides can be detected.

INTRODUCTION

Recently, a large number of new gangliosides has been isolated and identified in various tissues, including brain (for review, see 1-3). Minor components have been identified in mammalian brains (4-6) together with major components which were not separated from well known gangliosides in the common types of solvents used for thin layer chromatography (TLC) (7-16), even though more recent solvents give consistent improvements (17,18).

While many techniques are now available to separate gangliosides at a preparative scale, including column chromatography on silicic acid (19), silica gel (4,20), DEAE-cellulose (21,22) and DEAE-Sephadex (4-6), all analytical systems of TLC do not very well resolve the recently discovered complex population of gangliosides (4).

In an attempt to increase the efficiency of such separations, we developed a new solvent mixture which, using high performance TLC (HPTLC) gives very rapid, accurate and much more efficient separation of gangliosides. Presented are data on this solvent which show that the considerable heterogeneity of the ganglioside population found in rat cerebellum is not due to artifacts.

MATERIALS AND METHODS

Chemical

Standard gangliosides were obtained as previously described (18). HPTLC Silica Gel 60 plates (10 x 10 cm) and resorcinol were purchased from Merck AG (Darmstadt, GFR). Neuraminidase type VI from *Clostridium perfringens* was from Sigma Chemical Co. (St. Louis, MO).

Ganglioside Extraction from Rat Cerebella

Gangliosides were extracted by chloroform/methanol mixtures followed by phase partition according to Suzuki (13). Briefly, 1 vol of water and 12.7 vol of methanol were added to pooled fresh cerebella from rats of known ages and the tissue was homogenized. The mixture was stirred for 5 min at room temperature and 25.3 vol of chloroform was added. The suspension was stirred for another 10 min and centrifuged 30 min at 20,000 g and 20 C in stainless steel tubes. The pellet was then extracted with 20 vol of chloroform/methanol (1:2, v/v) by stirring 30 min at room temperature and centrifuging. Twenty vol of chloroform and then 16 vol of 0.88% (w/v) aqueous KCl were added to the pooled supernatant. The lower phase, washed with the previous vol of the theoretical upper phase (13), was discarded. The combined upper phases were evaporated to dryness in a rotary evaporator at room temperature.

For preparative purposes (1-10 mg of lipid-bound sialic acid), the dry material was suspended in a small vol of water and dialyzed for 3 days at 4 C against large volumes of distilled water. The residue obtained after lyophilization was taken up in chloroform/methanol (CM) (2:1, v/v) and stored at -20 C until needed.

For analytical purposes (where small amounts of gangliosides are involved), the dried

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upper phase was extracted in 1 ml CM (1:1) and centrifuged. The supernatant was evaporated to dryness in a stream of nitrogen, extracted with 200 μl of CM (1:1) and centrifuged. The clear supernatant was used directly for TLC analysis.

Analytical TLC

For this study, only HPTLC plates were used. Three previously published solvent systems and one new system were compared; solvent a: chloroform/methanol/aqueous KCl (17); solvent b: chloroform/methanol/NH_4OH (4); solvent c: methyl acetate/isopropanol/0.25% aqueous KCl (18) and solvent d: methyl acetate/*n*-propanol/chloroform/methanol/0.25% aqueous KCl (25:20:20:20:17, v/v).

With solvents a and b, HPTLC plates were run for 2 hr at 20 C and 1 hr with solvent c. With solvent d (developed by us to achieve better resolution of gangliosides), HPTLC plates were run for ca. 45 min at room temperature in a tank lined with filter paper until the solvent reached the top of the plate. Straight deposits of gangliosides, 5 mm long, in CM (2:1) were spotted with a glass capillary at 1 cm from the bottom of the plate and dried with a stream of cold air. Gangliosides were detected by the resorcinol/hydrochloric acid method of Svennerholm (23).

Preparative TLC of Cerebellar Gangliosides

Equal amounts of gangliosides (in terms of sialic acid) from cerebella of different ages (0, 4, 8, 12, 16, 20, 25, 30, 40 and 90 days) were mixed together. The HPTLC system was chosen for use at the semipreparative scale because of its ability to separate most of the individual gangliosides. The ganglioside mixture was spotted on the plate as an 8-cm-long streak and chromatographed in solvent d under conditions identical to those used for analytical purposes. After drying with a cold air stream, the plates were treated with iodine vapor and each ganglioside streak was lightly marked with a pencil. Traces of iodine were eliminated in a stream of cold air and silica gel was aspirated with a vacuum pump into capillary pipettes closed with glass wool. Material with the same R_f obtained from 6 different plates was recovered in the same pipette. Gangliosides were eluted first with CM (1:2) containing 5% water and then with methanol. The solvents were evaporated at room temperature in a stream of nitrogen. The dry residue was suspended in a small volume of CM (1:2) and centrifuged. The pellet was washed once and the pooled supernatants were dried in a stream of nitrogen and taken up in CM (2:1). The individual gangliosides were used in this form for carbohydrate analysis.

Ion Exchange Chromatography of Gangliosides

In 2 experiments, gangliosides were separated by ion-exchange chromatography on DEAE-Sephadex A 25 according to Momoi et al. (4). The system used was essentially the same except that column size (20 × 0.7 cm) and elution volume (600 ml) were reduced for the smaller quantities of gangliosides (10 mg of lipid bound NeuAc) used in our studies.

Treatment of Gangliosides by Neuraminidase

For the degradation of gangliosides by neuraminidase, the conditions of Ando and Yu (6), with regard to enzyme substrate ratio, detergent and temperature, were used. The incubations were performed in 0.2 M sodium acetate/acetic acid buffer, pH 5.0.

Gangliosides were extracted by adding 19 vol of CM (2:1) to the incubation mixture and clarified with filter paper previously cleaned with lipid solvents. The solution was dried in a stream of nitrogen at room temperature and was taken up in a small volume of CM (2:1).

Alkaline Treatment of Gangliosides

Two methods were used in alkaline treatment: alkaline methanolysis (6), and incubation for 2 hr at room temperature in the mixture *n*-propanol/isopropanol/25% NH_4OH (35:35:20, v/v) (24).

Carbohydrate Analysis

The carbohydrate composition of the isolated gangliosides was determined according to Zanetta et al. (25) by gas liquid chromatography (GLC) of the trifluoroacetate derivatives of the *O*-methyl-glycosides obtained after methanolysis.

RESULTS AND DISCUSSION

Efficiency and Properties of the New Solvent

As shown in Figures 1 and 2, the separation of all the standard gangliosides is achieved in less than 1 hr on HPTLC silica gel plates. G7, GM3, GM2, GM1, GD3, GD1a, GD2, GD1b, GT1b and GQ1 (and also spots corresponding to penta- and hexasialogangliosides) can be resolved in this system. The spots are very sharp and tailing is less pronounced than with other solvents for identical samples and quantities of gangliosides put on the plates. The

effect of salts (mainly KCl) is negligible and generally the dialysis step of the upper phase is unnecessary. Slight contamination by phospholipids does not modify the chromatographic separation nor the quantitative determination of ganglioside sialic acid on the plate by the resorcinol/hydrochloric acid method (23). The alkaline hydrolysis step of phospholipids generally used to purify the gangliosides extracts thus can be avoided. Only sucrose and glucosides (when present) can give rise to false determinations of ganglioside sialic acid in the R_f region close to GD1a. The separation is not greatly dependent on the temperature and generally no special precautions are necessary.

Heterogeneity of the Ganglioside Population of the Rat Cerebellum

As shown in Figure 1, the ganglioside profile of the rat cerebellum is much more complex than those published for the brain of most mammals, including man (either normal or pathological) and those of the rat cerebellum with other solvent systems. In solvent d, 16 spots (Fig. 1) with the typical color of sialic acid, revealed by the resorcinol-HCl methods (23), are detected. Isolation of each spot by preparative TLC and rechromatography indicate that such a complex picture is not due to chromatographic artifacts, provided that gangliosides are not submitted to drastic treatments during the various steps of isolation (see Methods).

Comparison with Standard Gangliosides

Parallel migration or comigration of total cerebellar gangliosides with standard gangliosides can suggest the structure of some of these compounds. However, as discussed later, such an identification is not at all sufficient. Spots with exactly the same migration as GM3, GM1, GD1a, GD1b, GT1b and GQ1 are present. G7 apparently is absent, whereas traces of GM2 (a maximum of 0.2% of total ganglioside sialic acid) were present. Two spots are detected between GM2 and GM1, with the slower migration appearing often as a diffuse band preceding GM1. Between GM1 and GD1a, 2 spots are evident, called GX1 and GX2. Two other unknown spots, GY1 and GY2, are localized between GD1a and GD1b. One other spot is detected between GD1b and GT1b called GZ. Below the GQ1 band, 2 minor spots can be detected when the plates are overloaded.

It should be mentioned that, between GM1 and GT1b, the background of the chromatographic plate is never unstained when a complex mixture of rat cerebellum ganglioside is submitted to TLC. When the space between

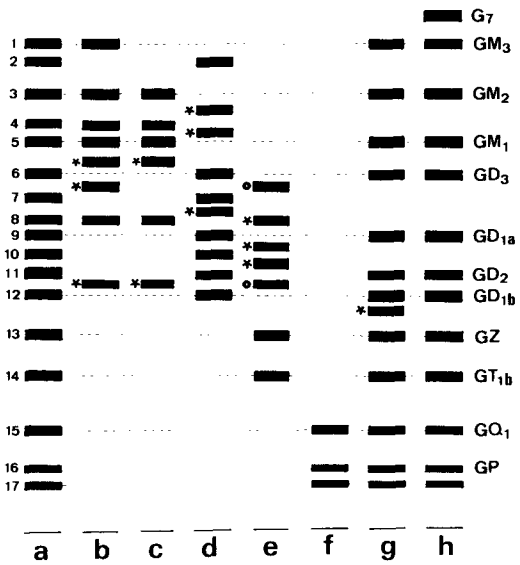

FIG. 1. Chromatography on HPTLC plates (10 X 10 cm) in solvent d of ganglioside extracts of cerebella of rats of different ages (0, 4 and 8 days old) revealed by the resorcinol-hydrochloric method (13). GX_1, GX_2, GY_1, GY_2 and GZ refer to unidentified gangliosides (see Results and Discussion).

each evident spot is eluted and rechromatographed, a spot with the same R_f is obtained. This observation strongly suggests that the background of the plate is not a result of tailing of some compounds with higher R_f but is from minor gangliosides.

DEAE-Sephadex Chromatography

The ammonium acetate gradient (4) permits the separation from a complex ganglioside mixture of 3 peaks, which should correspond (4), respectively, to mono-, di- and trisialogangliosides. The tail following the third peak contained gangliosides with a higher sialic acid content. A drawing of the TLC profiles of these peaks is given in Figure 2 whereas the proportion of each spot is indicated in Table I. These profiles were obtained both by comparison of the migration with the standard mixture and by cochromatography.

As shown in Figure 2b and Table I, the first peak of DEAE-Sephadex A 25 gave 8 spots. The spot migrating as GM1 is by far the most predominant. A spot migrating as GM3 is detected as well as one with the same R_f as GM2. GM1 never appears as a sharp spot but is preceded by a diffuse zone and followed by a sharp minor spot. Two other minor spots are detected with R_f values slightly higher than GD1a and GD1b, respectively. These 2 spots also are detected with solvents a and b.

In the second peak isolated by DEAE-Sephadex chromatography, numerous spots are detected (Fig. 2d). The first is found just below GM3. The second is found between GM2 and GM1 but is clearly distinct from the diffuse coloration before GM1 found in the first peak from the DEAE-Sephadex column. An intense spot is found below GM1 with a migration similar to that of GD3. Two other intense spots are detected which comigrate with GD1a and GD1b, respectively. It should be noted that 2 other less intense spots are seen between GD3 and GD1a and also between GD1a and GD1b. One of them has the same migration as GD2.

Such a complexity also is found with solvents, a, b and c, but it is less well defined than with solvent d. Thus, a total of 10 spots can be detected (Fig. 2d and Table I).

The third peak from the DEAE-Sephadex column gives 7 spots (Fig. 2e), 2 of them migrating between GD3 and GD1a, 3 between GD1a and GD1b and 2 below GD1b; the lower one migrates like GT1b.

The fourth peak of the DEAE-Sephadex column is composed of 3 slowly migrating spots (Fig. 2f). The most rapid spot migrates as GQ1 whereas the other 2 minor spots could be attributed to penta- and hexasialogangliosides.

These puzzling results are, in fact, in agreement with the results of Momoi et al. (4) on bovine gray matter. Even if the total ganglioside profile of bovine brain is much simpler than the cerebellar profile, these authors (using solvent b) found 4 monosialogangliosides, 7 disialogangliosides, 2 trisialo-, 1 tetra sialo- and 2 other unidentified spots. With the same solvent used for rat cerebellum, we found 6 spots for monosialo-, 8 for disialo-, and 5 for trisialogangliosides. In this system (solvent b), one disialoganglioside spot migrates as Q_Q1. This compound might correspond to the GB4 of Momoi et al. (4).

Alkaline Treatment

As shown in Figure 2g and Table I, alkaline treatments of a ganglioside mixture from rat cerebellum significantly modify the TLC profile of the mixture. Identical phenomena also have been observed by others (17,24). GM2 increases significantly whereas GX2 and GY1 almost completely disappear. However, a major spot which migrates below GD1b appears in a quantity identical to that of the decrease of GX2 spot. It can thus be postulated that GX2 contains a masked form of a ganglioside migrating in the zone of trisialogangliosides. In solvents a and b, this compound migrates as GD1b.

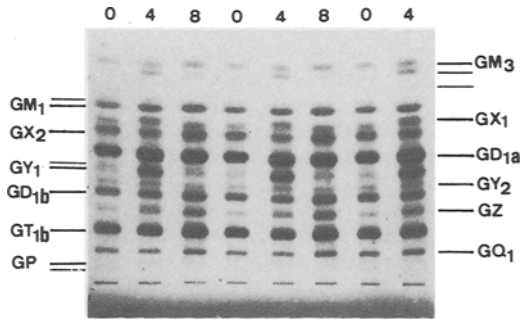

FIG. 2. Drawing of ganglioside profiles from pooled cerebella of rats of different ages obtained on HPTLC plates eluted with solvent d: (a) spots detected directly in the total ganglioside extract are numbered. Some of them were analyzed by gas liquid chromatography (see Table II); (b) profile of the gangliosides found in the first peak from DEAE-Sephadex chromatography (monosialogangliosides); (c) result of the action of neuraminidase on the total ganglioside extract shown in a; (d) profile of the gangliosides found in the second peak from DEAE-Sephadex chromatography (disialogangliosides); (e) profile of the gangliosides found in the third peak from DEAE-Sephadex chromatography (trisialogangliosides); (f) profile of the gangliosides found in the fourth peak from DEAE-Sephadex chromatography (tetra-, penta- and hexasialogangliosides); (g) action of alkaline hydrolysis on the mixture separated in a; (h) spots detected in the standard mixture of gangliosides. Gangliosides detected directly when the total mixture was submitted to HPTLC in solvent d are identified by numbers. Some compounds (* or °) appear only after separation of the ganglioside mixture on DEAE-Sephadex columns. The identification number is that of the spot of immediately higher R_f found by direct HPTLC.

Treatment with Neuraminidase

After treatment by neuraminidase from *Clostridium perfringens* in the presence of taurocholate (6), 6 spots are detected which have exactly the same migrations as the spots obtained in the first peak from DEAE-Sephadex chromatography. Polysialogangliosides and GM3 disappear and GM1 and GM2 increase (Fig. 2c). The spots with R_f values slightly higher than GD1a and GD1b persist even after a 72-hr treatment with the enzyme (Fig. 2c).

Isolation of Individual Gangliosides and Carbohydrate Analysis

As we have mentioned, individual gangliosides gave only one spot with the same migration as the initial ganglioside, if care was taken during isolation. However, heating during evaporation gave a pattern characteristic of degradation.

The results of carbohydrate analysis of individual gangliosides isolated by TLC in solvent d from a total mixture of gangliosides from rat cerebella of different ages (see Meth-

TABLE I

Percent Distribution of Sialic Acid in the Compounds Shown in Figure 2

Compound	Monosialo-gangliosides[a]	Disialo-gangliosides[a]	Trisialo-gangliosides[a]	Tetrasialo-gangliosides[a]	Direct TLC[b]	Direct TLC after alkaline hydrolysis[b]
1 (GM_3)	2.00	–	–	–	1.97	2.20
2	–	0.43	–	–	0.59	0.59
3 (GM2)	0.51	–	–	–	0.26	1.18
3*	–	0.15	–	–	–	–
4	0.92	–	–	–	0.59	0.59
4*	–	1.07	–	–	–	–
5 (GM1)	5.79	–	–	–	6.31	6.06
5*	1.12	–	–	–	–	–
6 (GD_3)	–	3.49	–	–	3.59	0.59
6*	0.15	–	–	–	–	–
6°	–	–	1.31	–	–	–
7	–	1.61	–	–	3.08	3.24
7*	–	2.34	–	–	–	–
8	0.51	–	–	–	–	–
8*	–	–	4.13	–	–	–
9 (GD_{1a})	–	20.65	–	–	27.70	29.76
9*	–	–	2.82	–	–	–
10	–	2.87	–	–	2.20	2.20
10*	–	–	7.04	–	–	–
11 (GD_2)	–	1.39	–	–	12.00	1.25
11*	0.97	–	–	–	–	–
11°	–	–	2.70	–	–	–
12 (GD_{1b})	–	8.59	–	–	8.76	8.79
12*	–	–	–	–	–	10.42
13 (GZ)	–	–	7.34	–	7.82	8.01
14 (GT_{1b})	–	–	22.10	–	21.78	21.72
15 (GQ_1)	–	–	–	3.50	3.25	3.50
16 + 17	–	–	–	0.5	0.50	0.56

[a]Results are expressed as percent of total sialic in the sample before fractionation on DEAE-Sephadex columns.

[b]Results are expressed as percent of total sialic acid recovered on the plates. For the nomenclature of the various spots, see Fig. 2.

ods) are given in Table II. As can be seen, the analysis agrees well with the expected structure of major spots with the exception of glucose (which cannot be accurately determined due to contamination from TLC plates) (26). Two gangliosides containing fucose can be detected: one monosialo-(5) and possibly di- or trisialoganglioside (Table II). The spot between GD1b and GT1b evidently is a trisialoganglioside which correlates with its behavior on DEAE-Sephadex. The possibility that the observed heterogeneity was due to different fatty acid or sphingosine composition was also tested on the same samples. As published previously (25), fatty acid methyl esters can be extracted by hexane after methanolysis and analyzed separately by GLC. We did not detect any significant differences in the fatty acid compositions of the spots we analyzed. The same also is true for sphingosine bases (analyzed together with sugars in our system) (18). The observed heterogeneity thus is essentially due to the carbohydrate part of the ganglioside population.

CONCLUSIONS

The solvent of choice in this study is a considerable improvement over other systems for the TLC separation of gangliosides (including much better resolution, reproducibility, insensitivity to salts and time-saving). However, it is evident (as shown previously by Momoi et al. (4) and Iwamori and Nagai [27]) that TLC techniques alone are insufficient for the separation and identification of a total ganglioside preparation from mammalian brain. By combining DEAE-Sephadex chromatography with our solvent system, up to 28 compounds could be resolved in rat cerebellum, compared to 16 revealed by TLC in our system without previous ion-exchange chromatography and only 8 in conventional TLC systems (solvent a).

Such inability of TLC systems to resolve a complex ganglioside mixture may lead to completely erroneous conclusions in certain studies of gangliosides. As shown in Table III, the ganglioside composition of adult rat cerebellum is dramatically dependent on the system used

TABLE II

Carbohydrate Composition[a] of Some Gangliosides of the Rat Cerebellum Isolated by Monodimensional TLC in Solvent d

Spot[b]	Expected structure	Fuc	Gal	GalNac	AcNeu	Other sugars[c] (GlcNac, Ma)
1	GM3	–	1.020	–	1.000	–
3	GM2	–	0.980	1.000	1.014	–
4		1.062	2.335	1.000	1.052	–
5	GM1	–	2.00	1.000	1.120	–
6	GD3	–	1.000	–	1.883	–
7		1.142	2.157	1.000	2.541	–
8		–	1.912	1.000	2.105	–
9	GD1a	–	1.968	1.000	2.000	–
10		–	1.501	1.000	2.3091	–
11	GD2	–	1.001	1.000	2.091	–
12	GD1b	–	2.147	1.000	1.927	–
13		–	1.925	1.000	2.873	–
14	GT1b	–	2.980	1.000	2.971	–
15	GQ1	–	1.964	1.000	3.868	

[a]Molar ratios.

[b]Same spot as in Fig. 2a.

[c]Glucose values are not shown because of the high contamination from HPTLC plates.

TABLE III

Comparison of Ganglioside Compositions[a] of Adult Rat Cerebellum

Ganglioside	From ref. 28	From ref. 29	From ref. 30	This study
GM3	–	5.80	1.9	1.17
GM2	–	1.41	0.4	–
GM1	3.07	11.95	7.6	4.24
GX1	–	–	2.0	1.06
GX2	–	–	–	0.05
GD1a	29.12	19.69	21.0	22.73
GY1	–	3.16	0.3	3.19
GY2	–	–	–	2.77
GD1b	20.69	21.10	12.0	7.15
GZ	–	–	–	17.47
GT1b	47.13	26.90	27.0	32.89
GQ	–	11.25	15.9	6.04
G (P+H)	–	–	–	0.37
GT1a	–	–	9.0	–

[a]Expressed as percentage of total sialic acid.

for the separation of these compounds. One major ganglioside (GZ) is completely missed with classical solvents since it is not separated from GD1b, the proportion of which appears to be much lower in our system. The same observation is true for GM3, GM1 and GQ1 because of a poor resolution of TLC systems. Such incomplete separations are particularly dangerous for developmental studies, since compounds with the same R_f values might correspond at different ages to different gangliosides. It is thus evident that considerable care must be taken in the identification of gangliosides when TLC systems with poor resolving power are employed. Work is now in progress to isolate and identify more precisely most of the gangliosides present in adult rat cerebellum.

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COMMUNICATIONS

Occurrence of Squalene in Skin Surface Lipids of the Otter, the Beaver and the Kinkajou

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ABSTRACT

In a previous survey of the skin surface lipids of 46 mammalian species, it was found that only 3, the otter, the beaver and the kinkajou, contained components with chromatographic mobility similar to squalene, and in each case, this material was the predominant constituent. The materials in question have now been isolated from each of these species and identified as squalene by thin layer chromatography, gas chromatography and infrared spectroscopy. The occurrence of squalene in the surface lipids of these particular species cannot be explained on the basis of either their evolutionary relationship or ecological niche.

INTRODUCTION

It has come to be recognized that the composition of sebum varies distinctly among species (1-3). For example, 2 of the major constituents of human sebum, triglycerides and squalene, comprising 50 and 12% of the mixture, respectively, have not been found in significant amounts in the skin surface lipids of other animals (1,2), although squalene has been reported to form 0.5 and 1.5%, respectively, of the skin surface lipids of the rat (4) and the mouse (5). It was therefore of some interest that, in a survey of the skin surface lipids of 46 species of mammals by thin layer chromatography (6), we observed that 3 species had a major lipid component with a chromatographic mobility like squalene. These species were the otter *(Lutra canadensis)*, the beaver *(Castor canadensis)* and the kinkajou *(Potos flavus)*. No indication of the presence of squalene was seen in the lipids from the 43 other species examined. We have now established that in the otter, the beaver and the kinkajou, squalene forms 44, 80 and 94%, respectively, of the surface lipids of these species.

METHODS AND RESULTS

Animals

The otter and 2 specimens of the kinkajou were from private animal collections. The beaver was kept at the Wildlife Research Station, Boone, IA. Fur samples were obtained from the animals with solvent-cleaned scissors, and the lipids were subsequently extracted from the fur with chloroform. Yields of total surface lipid (mg/g of fur) were: otter, 14; beaver, 3; and kinkajou, 30.

Analysis of the Lipids

Quantitative thin layer chromatograms. These were performed on aliquots applied in chloroform to 6-mm-wide lanes drawn in 0.25-mm layers of Silica Gel G. The chromatograms were developed successively with hexane (to 19 cm), toluene (to 19 cm) and hexane/ether/acetic acid (70:30:1, to 11 cm). After drying, the chromatograms were sprayed with 50% sulfuric acid and heated to 220 C to char the lipids. The charred chromatograms were then scanned with a photodensitometer to provide a record of the chromatograms (Fig. 1) and to allow quantification of the lipid mixtures by triangulation of the photodensitometric peak areas (7,8). The material subsequently identified as squalene formed 44, 80 and 94%, respectively, of the lipids from the otter, the beaver and the kinkajou.

Preparative thin layer chromatography. To obtain sufficient material for identification, aliquots of up to 60 mg of the surface lipids were applied to 1-mm layers of Silica Gel G and developed with hexane (to 19 cm). The chromatograms were then sprayed with 2′,7′-dichlorofluorescein and viewed under a UV lamp. The visualized lipid corresponding in mobility to squalene was removed from the chromatographic plate and recovered from the adsorbent with ether.

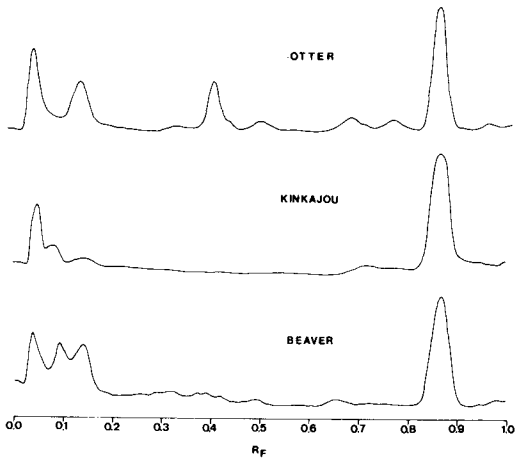

FIG. 1. Photodensitometer records of the skin surface lipids of the otter, the kinkajou and the beaver resolved on Silica Gel G by successive development in hexane (to 19 cm), toluene (to 19 cm) and hexane/ether/acetic acid (70:30:1, to 11 cm) and then charred by spraying with 50% H_2SO_4 and heating to 220 C. The material migrating to R_f 0.87 in each instance was identified as squalene. The material having R_f 0.0 to 0.05 is unidentified polar lipid and that at R_f 0.13 is free sterol, whereas those at R_f 0.65 to 0.8 are wax esters and diesters. The substance with R_f 0.4 in the lipids from the otter is probably glyceryl ether diesters.

Identification of Squalene

In the analytical thin layer chromatograms, the major constituent from each species had a mobility identical to that of squalene and produced the same color changes during charring. Corresponding fractions recovered from the preparative chromatograms were redissolved in hexane and subjected to gas chromatography at 215 C on a 3 ft x 1/8 in. id column containing 10% OV 17 on Chromosorb W. Each fraction showed a single component having a retention time identical to that of squalene. The identity of the 3 lipid specimens was confirmed by obtaining their infrared spectrum when applied to KBr discs. In each case, the spectrum was identical to that of authentic squalene (Sigma Chemical Co., St. Louis, MO).

DISCUSSION

Although the presence of squalene in skin surface lipids has previously been reported, it is only in human sebum that it has been found in appreciable concentration. Our finding of this lipid as the major component in the surface lipids of 3 other species serves to accentuate not only the diversity in the composition of skin lipids but also the unpredictability of their composition. The 3 species studied here are not closely related: 2 are carnivores, but belong to different families; the otter to the family Mustelidae (which includes the skunk, badger, mink, and ferret) and the kinkajou to the family Procyonidae (together with the raccoon and ring-tailed cat). Fourteen other carnivores, including those mentioned, showed no sign of squalene in their surface lipids. Of 13 rodents belonging to 7 families, the beaver was the sole species in which squalene was detected. Although the otter and the beaver have their aquatic environment in common, the kinkajou is an arborial carnivore which subsists principally on fruit. There is, therefore, no ready explanation, neither in the evolutionary relationships nor in the habitat, for the occurrence of squalene as the principal surface lipid of these 3 animals.

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Effect of Epinephrine and Dibutyryl Cyclic AMP on Δ5-Desaturation Activity of Rat Liver Microsomes

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ABSTRACT

The effect of epinephrine and dibutyryl cyclic AMP on the oxidative desaturation of [1-^{14}C]-eicosatrienoic acid to arachidonic acid of rat liver microsomes has been studied. Epinephrine, at a dose of 1 mg/kg/body weight, produced a significant decrease on Δ5-desaturation activity 3 hr after the injection. This effect was maintained up to 12 hr and reached the control values 48 hr after the hormone administration. Dibutyryl cyclic AMP treatment for 24 hr (5 mg/8 hr/100 g body weight) also produced a significant decrease of the conversion of eicosatrienoic acid to arachidonic acid in rat liver microsomes. The effect of epinephrine on Δ5-desaturation activity was postulated to be evoked through an increase of the intracellular concentration of cyclic AMP.

INTRODUCTION

The regulatory effect of several hormones on the activity of the Δ6- and Δ9-desaturases of rat liver microsomes is well established. It was previously demonstrated that diabetes depressed Δ6-desaturase activity (1-3) and that low doses of insulin enhance this reaction (1,4). In addition, hormones that increase blood glucose levels, such as glucagon, epinephrine, tyroxine and glucocorticoids depress the conversion of linoleic acid to γ-linolenic acid (5-8). On the other hand, Δ9-desaturase activity increases under insulin and tyroxine treatment (7,9).

The effect of hormones on Δ5-desaturation of fatty acids is little known, therefore, this research has been designed to investigate the effect of epinephrine and dibutyryl cyclic AMP (DB cAMP) on the activity of Δ5-desaturation of fatty acids.

MATERIALS AND METHODS

Adult female Wistar rats weighing 180-220 g and maintained on standard Purina chow were used. To study the effect of a pulse of epinephrine administration, rats were divided into groups of 4 animals each. They 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 injected subcutaneously with epinephrine at a dose of 1 mg/kg body weight. Animals were killed by decapitation 0.5, 1.5, 3, 12, 24 and 48 hr after the injection. Blood samples were taken to measure glucose levels using the o-toluidine method (10). The rats used as controls were treated identically, except that 0.9% saline was substituted for epinephrine.

In other experiments, the effect of dibutyryl cyclic AMP on Δ5-desaturation activity of rat liver microsomes was tested. The rats were assembled into 2 groups of 5 animals each. One group was injected intraperitoneally with dibutyryl cyclic AMP (provided by Sigma Chemical Co., St. Louis, MO) at a dose of 5 mg/8 hr /100 g body weight, and the other group, used as control, was injected with 0.9% saline solution. In this experiment, the animals were fasted for 24 hr, re-fed for 2 hr with Purina chow, and then killed 12 hr after the end of the feeding period that corresponded to 24 hr after the first injection.

Microsomes were separated by differential centrifugation at 100,000 × g as described previously (8). The desaturation of eicosatrienoic acid to arachidonic acid by liver microsomes was measured by estimation of the percentage conversion of [1-^{14}C] eicosa-8,11,14-trienoic acid (54.7 mC/mmol, 98% radiochemical pure; New England Nuclear Corp., Boston, MA) to arachidonic acid. Three nmol of the labeled acid and 97 nmol of unlabeled acid were incubated with 5 mg of microsomal protein in a shaker at 35 C for 20 min. The composition of the incubation medium and the assay procedure to measure the desaturation of eicosatrienoic acid was described elsewhere (8).

RESULTS AND DISCUSSION

The effect of the administration of a pulse of epinephrine on Δ5-desaturation activity of

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eicosatrienoic acid is shown in Figure 1A. Epinephrine markedly depressed this enzymatic activity, since 3 hr after the injection the effect was highly significant. However, the depression reached the lowest value at 12 hr. Δ5-Desaturation activity was then slowly recovered, and 48 hr after the injection it was similar to the control. Blood glucose also showed a fast response to the hormone. However, the increase of the blood glucose was found 30 min after epinephrine administration and recovered to control values after 24 hr. Therefore, the effect of epinephrine on the fatty acid desaturation was slower than the effect on glycogen breakdown.

Table I shows the effect of DB cAMP on Δ5-desaturation activity of rat liver microsomes. DB cAMP highly depressed the conversion of eicosatrienoic acid to arachidonic acid. Twenty-four hr after the treatment, the activity of the enzyme was reduced to less than one-half of the original value.

In a previous study, we demonstrated that epinephrine evoked a decrease of Δ6-desaturation activity on rat liver microsomes (6). From the results obtained in this experiment, it is evident that epinephrine also modifies the activity of Δ5-desaturase, since the conversion of eicosatrienoic acid to arachidonic acid markedly decreases after the catecholamine administration. However, the decrease of Δ5-desaturase activity was not as fast as that of Δ6-desaturase. While Δ6-desaturase decreased significantly 90 min after the injection of the hormone, Δ5-desaturase activity showed the effect after 3 hr, and the recovering of the enzymes' activities in both cases were similar (Fig. 1, A and B). The change in the concentration of cAMP evoked by epinephrine injection may be seen in Figure 1B.

The effect of epinephrine on Δ6-desaturation activity was postulated to be mediated through an enhancement of the intracellular cyclic AMP levels, since it was mimicked by pharmacological activators of β receptors and inhibited by β blockers (6,11). Moreover, DB cAMP administration to intact rats also produced a significant decrease in linoleic acid desaturation activity (5). The results obtained from this experiment with Δ5-desaturase would be produced by a similar mechanism. However, in a previous study, we did not find a significant change of the Δ5-desaturation activity after treating the rats with glucagon or DB cAMP (5). On the contrary, in the experiment described here, we could demonstrate that the conversion of eicosatrienoic acid to arachidonic acid was significantly decreased, not only by epinephrine, but also by DB cAMP

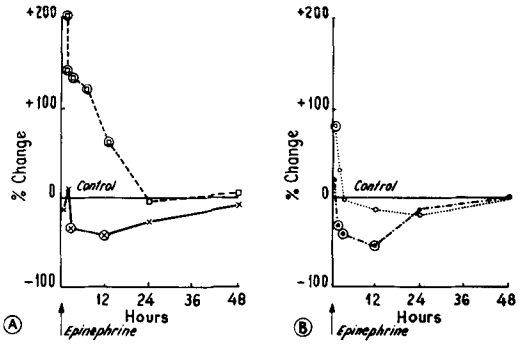

FIG. 1. (A) Effect of epinephrine administration on rat liver microsomal conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid (X—X) and plasma glucose levels (□----□). Zero point corresponds to the percentage conversion (average 22.0) for eicosatrienoic acid of normal rats injected with saline solution. Each point represents the average of 4 rats. In circles, results are significantly different from the controls, $P < 0.01$. (B) Effect of epinephrine administration on rat liver microsomal conversion of [1-^{14}C]-linoleic acid to γ-linolenic acid (·—·—·) and liver cAMP levels (o·····o). Zero points corresponds to the percentage conversion (average 21.7) for linoleic acid of normal rats injected with saline solution. Each point represents the average of 4 rats. In circles, results are significantly different from the controls, $P < 0.05$ for cAMP levels and < 0.01 for linoleic acid conversion to γ-linolenic acid. Results calculated from de Gómez Dumm et al. (6).

treatment. This apparent difference could be explained by the different experimental designs. In the experiment just mentioned, the rats were re-fed with a fat-free diet while they were simultaneously treated with DB cAMP, whereas in the present experiment, they were fed on Purina chow. It has been demonstrated that rats maintained on a fat-free diet show a significant decrease of the Δ5-desaturation activity when compared to animals fed a balanced diet (12). Therefore, the fat-free refeeding diet would have masked the decrease of Δ5-desaturation activity produced by DB cAMP

TABLE I

Effect of Dibutyryl Cyclic AMP on Δ5-Desaturation Activity of Rat Liver Microsomes[a]

	20:3→20:4	
	Conversion (%)	
Control	18.0 ± 1.7[b]	$P < 0.01$
DB cAMP	7.6 ± 1.1	

[a]See Materials and Methods for details.

[b]Averages of the analyses of 5 rats ± 1 standard error of the mean.

administration (5).

The results obtained in this work show that Δ5-desaturase activity of rat liver microsomes might be controlled by endocrinological factors. The effect of epinephrine on fatty acids Δ5- and Δ6-desaturases would be similar and it would be produced by an increase of the intracellular levels of cyclic AMP.

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Heat Activation of Rat Epididymal Fat Tissue Acetyl-CoA Carboxylase Is Due to Dephosphorylation by Its Endogenous Phosphatase[1]

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ABSTRACT

Acetyl-CoA carboxylase from rat epididymal fat tissue is activated by incubation at 30 C in the absence of citrate or metal ions. This activation is accompanied by a corresponding loss of ^{32}P from the labeled enzyme, and it is not blocked by the heat-stable phosphorylase phosphatase inhibitor proteins from rabbit muscle. We have succeeded in separating an activity which activates and dephosphorylates acetyl-CoA carboxylase from the carboxylase using polyethylene glycol-6000. These results suggest that the temperature-dependent activation of acetyl-CoA carboxylase in crude or partially purified preparations results from dephosphorylation of the carboxylase by bound phosphatase.

INTRODUCTION

Acetyl-CoA carboxylase, the rate-determining enzyme of fatty acid biosynthesis, is regulated by covalent modification: phosphorylation inactivates the enzyme and dephosphorylation activates it (1-3). Acetyl-CoA carboxylase has been observed to be activated by 3 mechanisms: polymerization by an agent such as citrate, cation-mediated activation, and the so-called "heat-activation" in the absence of either citrate or metal ions (4). Although it has been suggested that such a temperature-dependent activation might be due to polymer formation (4), even in the absence of citrate, the mechanism of such an activation has never been carefully examined. Allred and Roehrig have reported a 27,000 x g (20 min) fraction from liver which can be heat-activated and which is inhibited by a 105,000 x g (60 min), heat-stable factor that can be removed by Sephadex G-25 treatment (4). These results suggest that other mechanisms besides polymerization might be involved in heat activation.

In this communication, we report that acetyl-CoA carboxylase from rat epididymal fat tissue also can be heat-activated in the absence of citrate. This activation is accompanied by a corresponding loss of ^{32}P from labeled enzyme. The factor responsible for acetyl-CoA carboxylase activation has been separated from acetyl-CoA carboxylase and has subsequently been identified as acetyl-CoA carboxylase phosphatase. Unlike the activation and dephosphorylation of acetyl-CoA carboxylase by phosphorylase phosphatase (5), this activation is not inhibited by the rabbit muscle inhibitor proteins of phosphorylase phosphatase.

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MATERIALS AND METHODS

Chemicals

Acetyl-CoA, phenylmethylsulfonyl fluoride (PMSF), crystalline bovine serum albumin, fraction V bovine serum albumin (BSA), polyethylene glycol-6000, and ATP were obtained from Sigma Chemical Co., St. Louis, MO. Carrier-free [^{32}P]Pi and NaH[^{14}C]O_3 (59.3 mCi/mmol) were purchased from Amersham/Searle, Des Plaines, IL; NaH[^{14}C]O_3 was purified as described previously (1). Enzyme-grade ammonium sulfate was purchased from Schwarz/Mann; Orangeburg, NY; acrylamide, bis-acrylamide, and sodium dodecyl sulfate from Bio-Rad, Richmond, CA; glass fiber membranes from Reeve Angel, Clifton, NJ; complete Freund's adjuvant from Difco, Detroit, MI; Sephadex G-50 from Pharmacia, Piscataway, NJ.

Animals

Male Wistar rats weighing 210-255 g were raised in the departmental rat colony and fed ad libitum with a commercial animal diet.

Assay of Acetyl-CoA Carboxylase and Acetyl-CoA Carboxylase Phosphatase

Acetyl-CoA carboxylase was assayed after preincubating the carboxylase sample in a final vol of 100 μl, containing 20 mM Tris, pH 7.2, 1.0 mM DTT, 100 μg BSA, and 0.25 mM PMSF for the indicated length of time at 30 C. Following the preincubation, the assay was initiated in a final vol of 150 μl by addition of the following (final concentrations): 50 mM Tris, pH 7.2, 4.75 mM citrate, 5.5 mM $MgCl_2$, 1.4 mM DTT, 150 μg BSA, 0.16 mM PMSF, 2.0 mM ATP, 0.16 mM acetyl-CoA, and 13 mM $KH^{14}CO_3$ (1.5×10^6 cpm/assay). $^{14}CO_2$ incorporation into malonyl-CoA was measured

by the Majerus et al. method (6). One unit of acetyl-CoA carboxylase is the amount of enzyme which will form 1 μmol of malonyl-CoA per min at 30 C in the conditions just described.

Phosphatase activation is measured after adding fractions containing phosphatase activity to acetyl-CoA carboxylase and incubating at 30 C for the indicated length of time. After the incubation, acetyl-CoA carboxylase is assayed as already described.

Preparation of [^{32}P]-Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase was labeled by incubating finely minced adipose tissue with [^{32}P]phosphate as described previously (5). [^{32}P]Acetyl-CoA carboxylase was purified through the 45% ammonium sulfate precipitation as described previously (7), and was desalted with Sephadex G-50.

In experiments where the phosphatase was separated from acetyl-CoA carboxylase, the ammonium sulfate-precipitated enzyme was dialyzed into a buffer containing 100 mM phosphate, pH 7.5, 25 mM citrate, 0.5 mM EDTA, and 15 mM β-mercaptoethanol at 20 C. This enzyme was then slowly brought to 5% w/v with 50% polyethylene glycol-6000. After stirring for 3 hr at 20 C, the preparation was centrifuged at 27,000 x g for 10 min; the precipitate containing acetyl-CoA carboxylase was resuspended in 0.25 M sucrose, 50 mM Tris, pH 7.2, and 10 mM β-mercaptoethanol and was recentrifuged at 27,000 x g for 10 min to remove insoluble protein. The phosphatase was found in the supernatant fraction following polyethylene glycol fractionation; this fraction was centrifuged and dialyzed.

Enzymes

Purified rabbit muscle phosphatase inhibitor-1 (in its active, phosphorylated form) and inhibitor-2 were gifts from P. Cohen, University of Dundee (8).

Dephosphorylation of the Carboxylase

The determination of ^{32}P released during heat activation was carried out as follows: during the incubation of the Sephadex G-50-treated acetyl-CoA carboxylase at 30 C, 1.0-ml samples were taken at the indicated times. These samples were precipitated by 45% saturation with ammonium sulfate and resuspended in a buffer containing 50 mM Tris, pH 7.5, 0.15 M NaCl, 1 mM EDTA and 5 mM β-mercaptoethanol. Immunoprecipitation studies were carried out on these samples with antiserum to the fat tissue carboxylase, using 2.5 times the equivalence point of the antibody. The mixture was incubated 20 min at 30 C, then 16 to 20 hr at 4 C. The antibody-antigen complex obtained by centrifugation at 3,000 g for 15 min was then washed by resuspension and centrifugation 2 times with cold 0.15 M NaCl and 0.3 mM PMSF. The immunoprecipitates were suspended in a buffer containing 50 mM Tris, pH 7.2, 7% sucrose, 2% SDS, 5% β-mercaptoethanol, 0.5 mM EDTA, and 0.2 mM PMSF, and were heated in a boiling water bath for 15 min. These samples were subjected to 5% acrylamide SDS gel electrophoresis (9); the gels were sliced and the M_r = 220,000 carboxylase band was counted in an aqueous scintillation fluid.

RESULTS AND DISCUSSION

As shown previously, epididymal fat tissue acetyl-CoA carboxylase is activated rapidly upon preincubation at 30 C. The activation is complete within 15 min (Fig. 1). This activation is independent of both citrate and metal ions. To demonstrate that this heat-activation is due to a dephosphorylation of acetyl-CoA carboxylase, we prepared partially purified [^{32}P]acetyl-CoA carboxylase from fat tissue which had been incubated with ^{32}P for 3 hr as described in Materials and Methods. This enzyme was incubated at 30 C for the indicated length of time; 3 samples were taken at each of these times. One sample was used to assay the carboxylase by formation of [^{14}C]malonyl-CoA. A second sample was used to determine total ^{32}P. A third sample was precipitated with

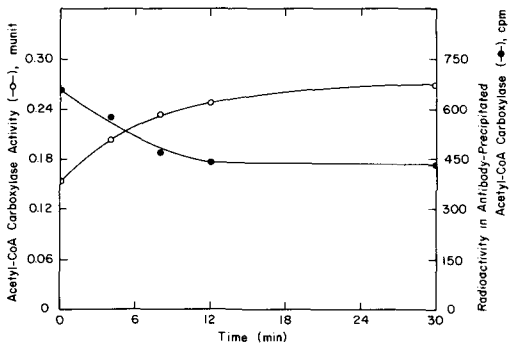

FIG. 1. Activation and dephosphorylation of fat tissue acetyl-CoA carboxylase by the endogenous phosphatase. The [^{32}P]labeled carboxylase was incubated for 30 min at 30 C to allow for the endogenous activation. At the indicated times, aliquots were withdrawn to assay the carboxylase (o—o) and to determine total ^{32}P content (•—•) in the M_r = 220,000 band of the SDS gel of the antibody-antigen complex as described in Methods.

antibody to the carboxylase. The enzyme-antibody complex was subjected to SDS gel electrophoresis, and the ^{32}P content in the carboxylase was then determined.

The experimental results relating the dephosphorylation and the carboxylase activation are shown in Figure 1. It is clear that the activation of the carboxylase is inversely related to the decrease in the amount of ^{32}P in the carboxylase itself.

Unlike the activation of acetyl-CoA carboxylase by phosphorylase phosphatase (5), this heat-activation is not inhibited by the rabbit muscle phosphorylase phosphatase inhibitor proteins. Table I shows that the addition of up to 4 units of these inhibitor proteins has no effect on the activation of acetyl-CoA carboxylase. Four units completely inhibited the activation of acetyl-CoA carboxylase by 3 munits phosphorylase phosphatase beyond the initial activation (5).

Hardie and Cohen reported that a factor which activates acetyl-CoA carboxylase can be removed if the carboxylase is precipitated with 5% polyethylene glycol (PEG) (10). Table II shows that acetyl-CoA carboxylase which has been treated in this manner does not undergo heat activation. However, if the 5% PEG supernatant is precipitated with 80% ethanol at 20 C as described by Brandt et al. for the preparation of phosphorylase phosphatase (11), the activating factor is found in the resuspended precipitate after dialysis. Further purification and characterization of this activating factor indicates that this factor is a phosphoprotein phosphatase. The details concerning the purifi-

TABLE I

Effect of Rabbit Muscle Phosphatase Inhibitor Proteins 1 and 2 on Activation of Acetyl-CoA Carboxylase

Inhibitor protein-1	Acetyl-CoA carboxylase activity	Inhibitor protein-2	Acetyl-CoA carboxylase activity
units	munits	units	munits
0	1.38	0	1.34
0.4	1.32	0.4	1.39
1.0	1.31	1.0	1.33
2.0	1.37	2.0	1.28
4.0	1.37	4.0	1.31

Ammonium-sulfate-treated acetyl-CoA carboxylase was activated by its endogenous phosphatase from 0.73 munits in the presence of increasing amounts of inhibitor proteins 1 or 2. After incubating for 15 min at 30 C in the absence of citrate, acetyl-CoA carboxylase was assayed as described in Methods.

TABLE II

Separation of Activator from Acetyl-CoA Carboxylase Using 5% Polyethylene Glycol Precipitation

Treatment	0 min preincubation activity	30 min preincubation activity
	munits	
Acetyl-CoA carboxylase after 45% ammonium sulfate	1.06	1.72
Acetyl-CoA carboxylase after 5% PEG precipitation + 20 μl ethanol-treated 5% PEG supernatant	1.19	1.38
40 μl ethanol-treated 5% PEG supernatant	1.17	1.71
60 μl ethanol-treated 5% PEG supernatant	1.19	1.96

Ammonium-sulfate-treated acetyl-CoA carboxylase was precipitated with 5% polyethylene glycol as described in Methods. The supernatant was then treated with 5 vol 95% ethanol at room temperature as described by Brandt et al. (11). Acetyl-CoA carboxylase activity is shown after 0 and 30 min preincubation at 30 C.

cation and properties of this phosphatase will be published elsewhere. These studies suggest that dephosphorylation by a phosphatase is involved in the heat activation of acetyl-CoA carboxylase.

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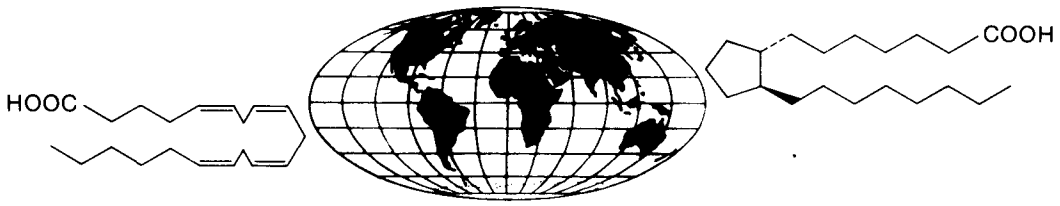
Golden Jubilee International Congress



on Essential Fatty Acids and Prostaglandins

(The week after the ISF/AOCS World Congress, approximately 410 persons attended the Golden Jubilee International Congress on Essential Fatty Acids and Prostaglandins, held at the University of Minnesota in Minneapolis. Jubilee President Ralph T. Holman and his program coordinators provided the following summary for JAOCS.)

In 1930 at the University of Minnesota in Minneapolis George O. Burr and M.M. Burr made the remarkable discovery that dietary linoleic acid was essential for growth, development and normal function in rats. At this time, pharmacological activity of a group of uncharacterized substances from semen and male accessory glands was also being described by workers in New York, England and Sweden. At the Karolinska Institute in Stockholm, U.S. von Euler first extracted and characterized this material as biologically active hydroxylated unsaturated fatty acid and named it "prostaglandin." There are now known to be several different prostaglandins of different degrees of unsaturation and different substituents.

World War II interrupted research on the "essential fatty acids" (EFA) and "prostaglandins" (PG), which in any case was then limited by inadequate technology. The 1950s saw the introduction of radioisotopes, gas liquid chromatography and mass spectrometry. During the 1950s and 60s EFA were shown to be required by several species, including man, and linoleic acid was found to be metabolized to longer chain polyunsaturated acids by a series of desaturation and elongation steps. Furthermore, the fatty acids derived metabolically from linoleic acid were considerably more potent as essential fatty acids.

In 1965, van Dorp and colleagues of Unilever in Holland and Bergström and colleagues at the Karolinska Institute in Sweden demonstrated that PG were enzymatically derived from arachidonic acid and other polyunsaturated acids. In the past decade, several new metabolites of arachidonic acid have been described, each with potent biological activity. These include the thromboxanes and leukotrienes described by Samuelsson and colleagues and prostacyclins described by Vane and Moncada. One or more of the original discoverers of all of the above findings actively participated in the Golden Jubilee Congress.

In spite of the evidence establishing a link between EFA and PG or other autopharmacological mediators, workers in both fields had remained separated. Pharmaceutical companies became excited by the potential of PG, their analogs and inhibitors in the fields of reproductive physiology, inflammation and thrombosis. Meanwhile, in the EFA camp, advances were made in establishing the role of EFA in cell membrane function, brain growth and the prevention of atherosclerosis and thrombosis. At the time, however, benefits of high linoleic acid diets to the cardiovascular system were largely attributed to reduction in

blood cholesterol.

Two years ago an international group investigated the feasibility of a marriage between the EFA and PG fields at a Golden Jubilee Congress to mark the 50 years that would soon have passed since their discoveries in 1930 and 1935. It was ascertained that Burr and von Euler (with a combined age of 160 years) were both able and willing to attend a congress as guests of honor and give addresses on their discoveries. With this focus, a scientific conference was organized in which new and exciting findings were presented against the historical backdrop of lectures by pioneers reviewing and updating their respective areas. It was obviously not possible to cover every topic in great detail, but the sessions were designed to highlight the precursor-product relationship between EFA, PG and other biologically important EFA metabolites.

A new feature of the programming was the final session on "Hot Stuff and Torrid Topics." Some subjects had been extracted from the offerings to the main program, whereas others were submitted at the meeting itself. The main criteria for acceptance in this session were that the data were completely new and the subjects were burning or controversial. The content of this session was kept a close secret throughout the meeting, and much in the way of speculation was overheard! This format was designed to generate excitement and to allow a plenary-type presentation to *any* investigator with something important to say.

The unique formula for this meeting worked in a very special way. It was a first meeting of two branches of science which had been distant acquaintances for many years. The knowledge each had to offer the other fused them into one and generated infectious enthusiasm. The essence of the meeting was the synthesis and dissemination of ideas and knowledge rather than an emphasis on personalities.

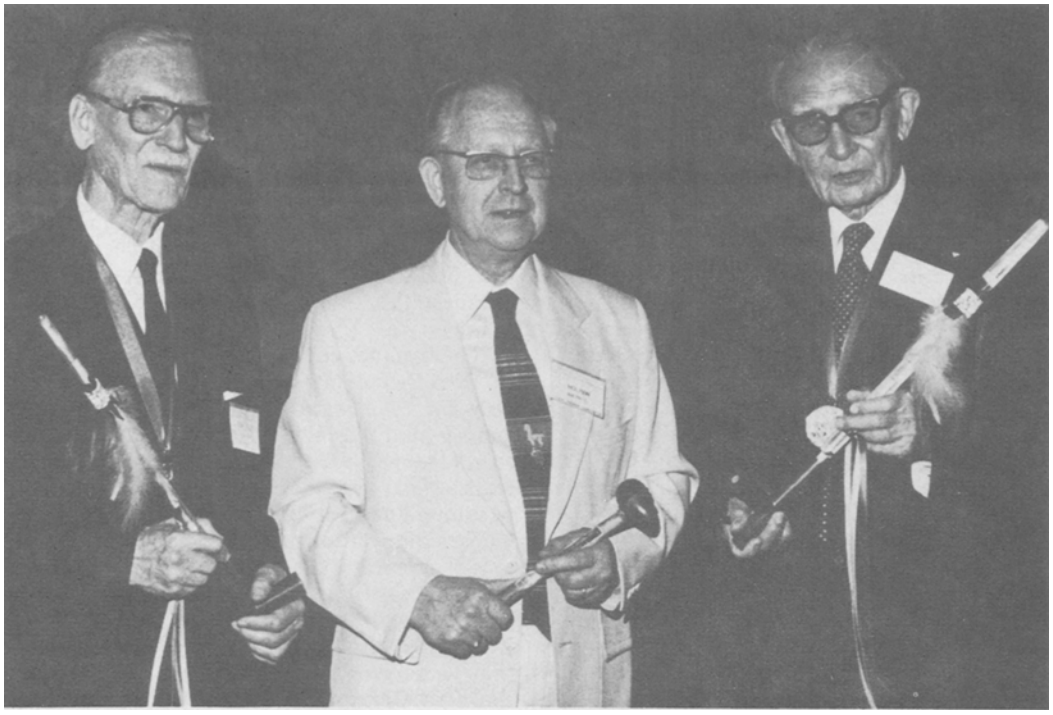
The reasons for the occurrence of polyunsaturated fatty acids in cell membrane lipids are now being elucidated. Studies of the rates of interconversion of EFA have led to the concept that nutritional management of the dietary precursors should affect biosynthesis of PG and other oxygenated products. Practical applications of this knowledge were reported at the congress. Retinopathy in human diabetics maintained for six years on a diet rich in linoleic acid has been prevented. Similarly, it now seems that in man the dietary intake of linoleic acid or of polyunsaturated acids of fish oils may reduce severity of hypertension and atherosclerosis.

Although dietary linoleic acid is converted to the PG precursors dihomo-γ-linolenic acid and arachidonic acid, individual oxidative metabolites of these EFA may have different or even opposing actions in several biological systems. Conventional descriptions of EFA deficiency as a scaly skin and an elevated triene/tetraene ratio may be inadequate because different disease states were described in which conversion of linoleic acid to the PG precursors is impaired. We now must also consider the spectrum of various PG, leukotrienes, thromboxanes and prostacyclins derived from the different polyunsaturated fatty acids ingested in different fats and oils. In this context, present recommendations for linoleic acid intake of 1-2% of dietary energy may have to be revised upwards, especially when one considers our present consumption of saturated fatty acids and arachidonic acid from animal fat.

If one considers that prostaglandins are themselves EFA, then psoriasis is one example of EFA-deficiency in which supplies of the linoleic and arachidonic acids are adequate but in which there is a block in the enzyme cyclooxygenase necessary for PG formation. In such cases replacement therapy with PGs or their synthetic analogs may be indicated.

New leukotrienes were described at the congress, bringing to three the families or series of leukotrienes. A double bond at the five-position seems obligatory for an unsaturated fatty acid to serve as a leukotriene precursor. Although no leukotrienes can be produced from 8,11,14-20:3, they can be produced from 5, 8, 11-20:3 (Mead's acid of EFA deficiency), from arachidonic acid 5,8,11,14-20:4 and from 5,8,11,14,17-20:5. Within each series of leukotrienes there are four individual members of which two have pro-inflammatory and bronchoconstrictor roles suiting them as mediators of inflammation and asthma. Probably these account for the biological activity of the slow reacting substance in anaphylaxis (SRS-A). There were also reports on chemical synthesis of novel leukotrienes and of the biosynthesis of leukotriene-like substances from 18-carbon fatty acids possessing Δ-5 unsaturation.

In the prevention of thrombosis and in the immune system, there was renewed speculation that PGE_1 and/or other derivatives of 8,11,14-20:3 may play previously hidden roles. The metabolic production of this acid from dietary linoleic and γ-linolenic acids, and its accumulation in free fatty acid pools may be involved in the process. Changes in PG metabolism induced by the therapeutic use of lidocaine in endotoxin shock in baboons indicated that



U.S. von Euler, (left), pioneer prostaglandin researcher, and George O. Burr (right), pioneer EFA researcher, pose at Golden Jubilee International Congress with conference president Ralph T. Holman. Peace pipes were presented to the two men to symbolize their contributions to knowledge.

prostacyclin might be involved in protection against shock. This finding, for the first time, provided a rational link between adequate nutrition and susceptibility to stress and shock. The concept that prostacyclin is a circulating hormone came in for heavy attack from several groups who had used various assay methods to test the possibility, and there was some mention of the original findings being artifactual.

The torrid topic session produced a threat to the postulated role of thromboxane A_2 in platelet thrombus formation. Although thromboxane A_2 is more potent as a platelet aggregator than the endoperoxides (PGG_2 and PGH_2) from which it is enzymically derived, these endoperoxides may also produce aggregation in their own right. Work was described in which a specific thromboxane inhibitor was used to block thromboxane production induced by arachidonic acid, adrenaline and collagen, but aggregation was unimpaired even though an antagonist of endoperoxide-induced aggregation could inhibit aggregation induced by these agents.

Another torrid topic was columbinic acid, derived from seeds of the Columbine. Its structure is similar to linoleic acid with a *trans* double bond at the five-position. This acid was found to express many EFA functions associated with linoleic acid, but it cannot be converted to prostaglandins. The new essential fatty acids thus provided an experimental tool for separating PG-mediated functions from the structural lipid functions of essential fatty acids. For instance, in rats, columbinic acid could restore to normal the scaly skin lesions of EFA-deficient rats, but could not normalize the response to inflammation. Kidney lesions and defective platelet function also were not restored.

Recently, much publicity has been given to the view that we should protect ourselves against thrombotic disease by subsisting on an Eskimo diet, whose effects seem mainly attributable to its high content of eicosapentaenoic acid (EPA), which is a precursor for PG of the 3-series. However, from evidence presented at the congress, this view received some setbacks. There were several reports that fish oils containing EPA could indeed prolong bleeding and inhibit platelet aggregation, but sometimes severe reductions in platelet count were encountered. Furthermore, available evidence from animal and human studies did not support

the view that EPA owes its antithrombotic actions to its conversion to a prostacyclin-like PGI_3 by enzymes in the vasculature. Feeding studies with EPA-rich fish oils in both animals and a human volunteer showed evidence of reduced platelet thromboxane production, and also of reduced production of prostacyclin, which has antithrombotic properties. The question arose whether diets rich in EPA cause undesirable increases in leukotriene/prostaglandin ratio. In a human volunteer who subsisted on a severe Eskimo diet for a prolonged period, some changes were seen that male members of the audience did not wish to share! Clearly additional animal studies with pure EPA, followed by closely monitored human volunteer studies, are needed.

Perhaps the most exciting of all the torrid topics was a report suggesting that arthritis may be alleviated by dietary means involving 6,9,12-18:3. This result was found in an animal model of immune-mediated arthritis and the leukocytes from such animals were not able to take part in chemotaxis. With this and the other reported examples from the immune response and cardiovascular areas, the distinct impression was given that we may be on the verge of new and exciting developments in dietary control of many disease states. This goal might be more easily realized if the suitably chosen dietary adjunct is combined with a drug which specifically redirects oxidative metabolism of EFA and other polyunsaturated fatty acid precursors. Alternatively, or in addition, a drug could be administered that blocks cyclic AMP degradation by phosphodiesterases. Such a drug should enhance desirable antithrombotic actions of PG whose formation is enhanced by dietary means. As reported at the meeting, antithrombotic properties of all PG are exerted through stimulation of cyclic AMP production in platelets. Certainly, pharmaceutical companies involved in development of drugs whose actions impinge on PG/thromboxane/leukotriene-mediated mechanisms should be aware of the importance of dietary EFA in ultimately controlling actions of these drugs. Dietary factors should be accurately monitored in both animal tests and clinical studies.

It was part of the historical picture that G.O. Burr and U.S. von Euler should meet for the first time at this congress, and it was appropriate that the EFA and PG people should also meet for the first time with such fertile results. Dr. Ralph T. Holman, the Congress Chairman, reminded us (with dry wit) of two other people who also met in the land of 10,000 lakes–Hiawatha and Minnehaha. Because parodies of the Song of Hiawatha had been part of the literature of lipid researchers for two decades, the congress was opened and closed in that style.

The banquet ended with two historic speeches from two remarkable men–remarkable not only for their work but for their great insight and natural humility. As scientists we were reminded to "expect the unexpected" and to realize that original discoveries often only become important after development by others whose contributions are often forgotten.

To celebrate the occasion in a more substantive way, the congress had medals struck which bore the images of Burr and von Euler. Burr and von Euler were renamed Hiawatha, they were given their gold medals for winning the lipid Olympics and they were given Chippewa peace pipes symbolic of their wisdom and honor. Replicas were also presented to Drs. Sune Bergström, David van Dorp, Jan Boldingh, Hugh Sinclair, the program coordinators of the congress, and the Department of Biochemistry of which Burr was once head, all of whom had done so much to stimulate awareness of the importance in man of dietary essential fatty acids and prostaglandins. All attendees at the banquet received replicas in pewter.

Then the Fellowship of Hiawatha
Left the cavern, wended homeward,
But they left with inspiration
From the council halls of learning,
From the speeches of the Four Winds
From the Wisdom they had gathered
At the feet of Hiawatha.

R.T. Holman, M. Crawford,
J.F. Mead, A.L. Willis
Coordinating Committee