Lipid Composition of Beef Heart Ventricle

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ABSTRACT

The lipid class composition of beef heart ventricle was determined by a combination of diethylaminoethyl cellulose column chromatography and quantitative thin-layer chromatography. Percentages of the total lipid were: triglyceride, 43.6; cholesterol, 7.4; phosphatidyl choline, 22.8; sphingomyelin, 4.0; phosphatidyl ethanolamine, 11.2; diphosphatidyl glycerol, 5.8; phosphatidyl serine, 1.2; phosphatidyl inositol, 3.0; phosphatidyl glycerol, 0.9; unsaturated hydrocarbon, 0.02; saturated hydrocarbon, 0.20. Nonlipid components represented 33.6% of the crude chloroform/methanol extracts.

INTRODUCTION

ALTHOUGH THE HEART is an important organ and heart mitochondria have been the subject of numerous investigations, little information is available on the lipid composition of heart (1–5). This relative lack of information led to the following investigation, the first quantitative analysis of heart lipid class composition employing a diethylaminoethyl cellulose column chromatographic procedure.

MATERIALS AND METHODS

Solvents and Nitrogen

All solvents were reagent grade redistilled from glass, and prepurified nitrogen (less than 7 ppm oxygen) was used.

Extraction of Lipids

Fresh beef heart obtained from the slaughterhouse was freed of adipose and connective tissues and passed through an electric meat grinder four times to obtain a homogeneous paste. Extraction of lipids was accomplished with 100-g portions of the paste with chloroform/methanol 2/1 in a nitrogen atmosphere (6). The crude chloroform/methanol extract was evaporated to dryness in a 2-liter round bottom flask and then thoroughly dried in a vacuum desiccator over KOH. When the crude lipid was extracted with chloroform/methanol 2/1 to free of denatured protein, only a portion of the protein was present as a sufficiently granular precipitate to be readily removed by filtration in contrast to proteins of crude lipid extracts of brain. Repeated evaporations with chloroform/methanol 2/1 to which enough water had been added to give a slight turbidity, followed by drying over KOH in a desiccator, were necessary before all of the protein had been denatured and could be removed by filtration. The alternate evaporation and drying were carried out until no turbidity was observed upon treatment with chloroform/methanol 2/1. The lipid thus prepared was free of protein and good recovery (100±2%) of the solids applied to DEAE columns was obtained.

Attempts were made to evaporate the final chloroform/methanol 2/1 filtrate free of protein to a small volume, transfer to a mortar, evaporate to dryness under a vacuum in a nitrogen atmosphere, and mix the lipid residue in a mortar with a pestle as for brain lipids (6). These operations were not conveniently performed as with brain lipids since heart lipid became moist while being mixed in the mortar. Representative samples and reproducible weights for column chromatography were obtained by solution of lipid in chloroform or a chloroform/methanol mixture, transfer of aliquots to 50-ml side-arm filtering flasks, evaporation of solvent under nitrogen, and drying to constant weight over KOH.

DEAE Column Chromatography

DEAE column chromatography was carried out as previously described (6) using a 4.5 (I.D.) × 20 cm column packed with 45 g of DEAE cellulose (Selactacel DEAE, regular grade, Brown Company, Berlin, N. H.). The chromatography tube was equipped with a 1-liter solvent bulb, a Teflon stopcock, and a glass wool plug to retain adsorbent.

The DEAE column was packed as a slurry in glacial acetic acid and acetic acid washed out with methanol (3 bed volumes). The column was washed with 5 bed volumes each of chloroform/methanol 1/1, chloroform/methanol 9/1, and chloroform. Column performance was tested with cholesterol (8). This test discloses any channels that may be present in the column and gives a good estimate of the column volume

Beef heart lipid (200–300 mg) was applied

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TABLE I
DEAE Column Chromatography of Beef Heart Lipids

Solvent	Volume (ml)	Weight (mg)	% Total extract	Substance(s)
C/M 9/1	1800	165.6	55.7	Ch, TG, PC, Sph
C/M 7/3	1550	26.35	8.8	PE-form I
CH_3OH	1250	42.40	14.2	Water solubles, and PE-form II
C/HAc 3/1	1700	20.30	6.8	U, PE-form II
HAc	1000	8.75	2.9	PS, U
C/HAc 3/1 + 0.01 M NH_4Ac	3000	28.55	9.6	DPG, PI, U
CH_3OH	1000	1.05	0.3	U
C/M 4/1 + 20 ml/l NH_3	1500	4.25	1.4	PI, traces U
C/M 4/1 made 0.01 M in NH_4Ac + 20 ml/l NH_3	1500	1.85	0.6	PI, PG, U
CH_3OH wash	1000	5.85	1.9	U
HAc wash	1000	0.90	0.3	U

Abbreviations: C/M = $CHCl_3/CH_3OH$; HAc = acetic acid; Ch = cholesterol; TG = triglyceride; PC = phosphatidyl choline (all forms); Sph = sphingomyelin; PE = phosphatidyl ethanolamine (diacyl, plasmalogen, and alkoxy forms); PI = phosphatidyl inositol; PG = phosphatidyl glycerol; U = uncharacterized nonlipid; PS = phosphatidyl serine (all forms); DPG = diphosphatidyl glycerol (cardiolipin); NH_3 = concentrated (28% by weight) aqueous ammonia.

to columns in 10 ml of the first eluting solvent. Columns were eluted first with chloroform followed by chloroform/methanol 9/1 or immediately with chloroform/methanol 9/1 when triglycerides and cholesterol were to be eluted along with phosphatidyl choline and sphingomyelin. Fractions of 40 ml were collected at a flow rate of 10 ml/min. The course of elution was followed with the solids and ninhydrin tests (7) through the methanol eluate. Following this, all fractions were collected in bulk. The solvents, elution volumes, and compositions of the fractions are given in Table I.

Fractions were evaporated to dryness in a rotary evaporator first flushed with pure nitrogen (6). Samples were then transferred to 50-ml side-arm filtering flasks, the solvent removed under nitrogen on a vacuum rack (6), and the samples dried over KOH in a vacuum desiccator and weighed. The fractions were then dissolved in chloroform or chloroform/methanol 2/1 at a concentration of 2–5 mg per milliliter and spotted for quantitative thin-layer chromatography (TLC).

Thin-Layer Chromatography

TLC was carried out essentially as described by Rouser et al. (9) with an adsorbent composed of 9 parts of Silica Gel Plain (Warner-Chilcott Laboratories, Instrument Division, Richmond, Calif.) plus 1 part magnesium silicate (Allegheny Industrial Chemical Co., P.O. Box 786, Butler, N. J.). Various chloroform/methanol/water mixtures (65/25/4, 65/35/5, 60/35/8) as well as 1-butanol/acetic acid/water (60/20/20) were used as developing solvents depending upon the substances to be determined.

Glass plates (20 × 20 cm) were spread with a water slurry of adsorbent (about 20 g of adsorbent mixed with 65 ml of water to spread five plates). After spreading, the adsorbent layer was heat activated for 20 min at 120C, the plates cooled in air for 30 min, spotted and chromatograms developed with the desired solvent. The air-dried plates were sprayed with the sulfuric acid-potassium dichromate reagent (1.2 g $K_2Cr_2O_7$ in 200 ml 55% by weight H_2SO_4). Spots were developed at 180C for 30–60 min. The plates were cooled, the back sides carefully cleaned, and the density of the sample spots and spots from standards determined with a Photovolt densitometer (Photovolt Corp., 1115 Broadway, N. Y.) using a recorder and integrator. Spotting for TLC was by the single spot or row of small spots techniques. Four applications each of sample and standard were made per chromatogram and a minimum of two chromatograms were used for each analysis.

Various pure lipid standards for quantitative TLC were prepared by column chromatography, largely from a beef brain (6,10). Commercially obtained cholesterol recrystallized from ethanol and a pure sample of triglyceride isolated from beef spleen were also used as standards.

RESULTS

The total lipid (free of nonlipid) of beef heart ventricle varied between 2.7 and 3.7% of the fresh weight of the organ. Table I shows the details of the elution of different fractions of beef heart lipid from DEAE columns and the percentages by weight represented by each fraction. Table I shows that more than 50% of the sample is eluted in the first two fractions. The crude lipid extracted with chloroform/methanol 2/1 contains a large amount of water-soluble nonlipid material (5). The nonlipids appear in all fractions except the chloroform and chloroform/methanol 9/1 fractions. The presence of these substances makes quantitative determination of the lipid classes in each fraction by TLC essential. The amounts of the various lipid classes of beef heart lipid are given in Table II. The values are averages of four separate analyses on each

TABLE II
Lipid Composition of Beef Heart

Lipid Class	Percent C/M 2/1 extract	Percent total lipid [a]	Molar ratios of phospholipids: Present study	Marinetti et al. (1) (pig heart)	Youngs and Cornatzer (2) (beef heart)	Gray and Macfarlane (2) (beef heart)	Wheeldon et al. (4) (beef heart)
Triglyceride	28.9	43.6	—	—	—	—	—
Cholesterol	4.9	7.4	—	—	—	—	—
Phosphatidyl choline	15.0	22.8	46.5	40.4	33.2	42	50.2
Phosphatidyl ethanolamine	7.4	11.2	22.8	21.1	20.1	38	32.3 [b]
Form I	5.6	—	—	—	—	—	—
Form II	1.8	—	—	—	—	—	—
Phosphatidyl serine	0.8	1.2	2.5	3.6	13.3	5	—
Phosphatidyl inositol	2.0	3.0	6.1	9.5	13.4	2	2.3
Phosphatidyl glycerol	0.6	0.9	1.8	—	—	—	—
Diphosphatidyl glycerol	3.9	5.9	12.0	11.4	—	9	10.1
Sphingomyelin	2.9	4.0	8.2	9.5	19.8	5	5.1
Nonlipid	33.6	—	—	—	—	—	—

[a] Excluding nonlipid.

[b] Phosphatidyl ethanolamine plus phosphatidyl serine.

of two extracts prepared from one ventricle and one analysis of a second ventricle. Individual values differed by no more than 6% from the mean values.

The lipid classes eluted with chloroform/methanol 9/1 are triglyceride, cholesterol, phosphatidyl choline, and sphingomyelin. Triglyceride and cholesterol (as well as other minor neutral lipids) are elutable from DEAE columns with chloroform and can therefore be separated from phosphatidyl choline and sphingomyelin (6). This is not particularly advantageous when the DEAE-TLC procedure is used for quantitative estimation since all of these lipid classes are readily separated by TLC and can be determined by the charring technique.

Phosphatidyl ethanolamine and some nonlipids are eluted from DEAE with chloroform/methanol 7/3. Phosphatidyl ethanolamine eluted with chloroform/methanol 7/3 is designated as PE-form I. The phosphatidyl ethanolamine in this fraction can be removed from almost all of the nonlipid material in the fraction by solution in cyclohexane. The remaining nonlipid material is soluble in chloroform/methanol 2/1. The nonlipid substances do not give spots on TLC with the charring or rhodamine 6G reagents for lipids.

Methanol used as eluting solvent after chloroform/methanol 7/3 elutes primarily water soluble nonlipids and a small amount of a different form of phosphatidyl ethanolamine. The phosphatidyl ethanolamine eluted with methanol is designated as PE-form II because its binding properties to DEAE are different from that of PE-form I. PE-form II is only partially eluted with methanol. The remainder of this form is eluted with chloroform/acetic acid 3/1. If methanol is not used as eluting solvent before chloroform/acetic acid 3/1, all of PE-form II is eluted with the latter solvent. The chloroform/acetic acid fraction contains nonlipid and a trace of free fatty acid in addition to PE-form II. PE-form II has the same elution characteristics as PE-form I on silicic acid columns and TLC migration of both forms is the same.

Glacial acetic acid eluted phosphatidyl serine and some uncharacterized nonlipid substances. Phosphatidyl serine was determined by quantitative TLC and nonlipid materials in the acetic acid fraction were estimated by difference. Diphosphatidyl glycerol, a part only of the phosphatidyl inositol present, and uncharacterized nonlipids were eluted with chloroform/acetic acid/ammonium acetate. Chloroform/methanol/ammonia and chloroform/methanol/ammonia/ammonium acetate elution of DEAE columns removed the remainder of the phosphatidyl inositol and some uncharacterized components.

DISCUSSION

Lipids are recognized to be important constituents of biological membranes and most if not all of the polar lipid appears to be present in membranes. The determination of the lipid composition of whole beef heart is of interest

to compare with that of beef heart mitochondria (9,11). The molar ratio diphosphatidyl glycerol/phosphatidyl ethanolamine/phosphatidyl choline is 1/2/2 for mitochondria and 1/2/4 for whole heart. The phospholipid values obtained in this laboratory are in relatively good agreement with those reported by Marinetti et al. (1) for pig heart ventricle, but are very different from values for beef heart ventricle reported from three other laboratories (Table II). The large differences noted for beef ventricle when silicic acid impregnated paper (3) or silicic acid column chromatography (2,4) are used for analysis cannot be explained fully, although the decomposition of lipids on silicic acid columns observed in one laboratory (2) can explain, at least in part, the different values reported.

Extraction with chloroform/methanol 2/1 yields much protein indicating that much of the lipid is extracted as proteolipid (lipid-protein complex). In order to dissociate the lipid from the protein, the residue obtained after evaporation of the crude lipid extract was thoroughly dried, taken up in chloroform/methanol 2/1, insoluble denatured proteins removed by filtration, and the filtrate evaporated to dryness. The process was repeated several times to denature the protein completely and to obtain lipids quantitatively in the free form. Addition of water to the chloroform/methanol 2/1 was found to aid in the denaturation of the protein. The presence of proteolipid in heart and a procedure for isolation has been reported by Murakami, Sekine and Funahashi (13). Proteolipid protein can be removed quickly from lipids by Sephadex column chromatography (5,12). Autoxidation of lipids during repeated extraction, filtration, and evaporation was reduced by performing all operations under nitrogen. The crude solids obtained by chloroform/methanol 2/1 extraction were found to be hygroscopic and accurate sample weights for column chromatography were obtained only with care to avoid errors related to this property. Heart lipid freed of water-soluble nonlipid by Sephadex column chromatography does not show this property.

Evidence for the presence of sterol esters was sought by silicic acid column chromatography, but no trace of esters of cholesterol or other sterols was found. Beef heart was found by silicic acid chromatography in the present studies to contain a minute trace (0.02%) of unsaturated hydrocarbon (probably mostly squalene) and a trace (0.20%) of saturated hydrocarbons. These hydrocarbon fractions were isolated quantitatively from a silicic acid column prepared in and eluted with n-hexane.

It will be noted in Table II that about 25% of the total phosphatidyl ethanolamine in beef heart is present in an uncharacterized form (form II) that may be an artifact of binding of a portion of the phosphatidyl ethanolamine through some nonlipid component.

There is a marked difference in the lipid composition of beef heart and beef brain (6), particularly with regard to the amounts of cholesterol, glycolipids, and some phospholipids. Beef brain contains a great deal of cholesterol, cerebrosides, sulfatides, and sphingomyelin but does not contain triglyceride. Heart contains much triglyceride, a low level of cholesterol, no cerebroside or sulfatide and a low level of sphingomyelin. These differences are reflections of major differences in membrane structures in these two organs.

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Agents Affecting Lipid Metabolism. XXVI. Specificity of Some Inhibitors of the Late Stages of Cholesterol Biosynthesis [1]

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ABSTRACT

The capacity of 22,25-DAC,[2] AY-9944 and triparanol to inhibit cholesterol biosynthesis from three precursors, mevalonate, 7-dehydrocholesterol and desmosterol, has been studied in rat liver homogenates.

Evidence is presented that, in vitro, 22,25-DAC, a potent inhibitor of the sterol Δ^{24}-reductase, also inhibits the 7-dehydrocholesterol-Δ^{7}-reductase system.

INTRODUCTION

IN THE PAST FEW YEARS we have been searching for novel types of inhibitors of cholesterol biosynthesis (1-8). The basic testing procedure consisted of determining the effect of a given compound on the incorporation of 2-C^{14}-MVA into cholesterol by a rat liver homogenate. At that time it became known that a factor limiting the therapeutic usefulness of triparanol, an extensively studied inhibitor of the Δ^{24}-reductase, was the accumulation of desmosterol (24-dehydrocholesterol), a sterol biosynthesized instead of cholesterol in the presence of triparanol (9). In view of this, a compound found by the test to block incorporation of MVA into cholesterol, was in turn tested in vitro for its effect on the 24-reductase system as reflected in its effect on the conversion of 26,27-C^{14}-desmosterol to cholesterol (6).

Application of this screening procedure has led to the discovery of inhibitory activity in DBED (7), a finding which has eventually led to the synthesis of AY-9944 (10), a very potent, orally active inhibitor of cholesterogenesis (4). AY-9944 was the first agent shown to act by inhibiting the Δ^{7}-reductase system (4,11-13). Subsequently, any compound inhibiting cholesterol formation from MVA was also tested for its effect on the Δ^{7}-reductase.

Previous studies with 22,25-DAC revealed the appearance of desmosterol in tissues of laboratory animals (3,14). Subsequently, the direct measurement of the effect of 22,25-DAC on the Δ^{24}-reductase system in vitro (6) has demonstrated that its predominant mechanism of action consisted in the inhibition of the enzymatic reduction of the $\Delta^{24,25}$ double bond in the side chain of sterols. We now wish to report on the findings of a second site of inhibition of hepatic cholesterogenesis by 22,25-DAC. The inhibitors AY-9944 and triparanol were used as reference standards.

EXPERIMENTAL PROCEDURE

2-C^{14}-MVA (Nuclear Chicago Corp.), 26,27-C^{14}-desmosterol (Volk Biochemical Co.) and 7-dehydrocholesterol (Mann Research Laboratories, Inc.) were used as substrates. Commercially available 7-dehydrocholesterol is usually contaminated with its 5α,8α-peroxide (13) (see below); before use, it was crystallized twice from ethyl acetate-methanol (15). The inhibitors used were triparanol (a gift from C. A. Bunde, Wm. S. Merrell Co.), 22,25-DAC (a gift from V. A. Drill, G. D. Searle and Co.) and our AY-9944.

The effect of these inhibitors on the incorporation of 2-C^{14}-MVA and 26,27-C^{14}-desmosterol into cholesterol in rat liver homogenates was determined as described previously (6). Radioactivity measurements were made using a Nuclear Chicago Model 720 Liquid Scintillation Counting System.

Δ^{7}-Reductase inhibition was assayed by a modification (7) of the original procedure of Kandutsch (16), which consists in measuring the disappearance of 7-dehydrocholesterol in the presence of liver enzymes and suitable cofactors. The homoannular $\Delta^{5,7}$-diene system of 7-dehydrocholesterol is susceptible to Diels-Alder-like addition of oxygen to form the ultraviolet transparent transannular 5α,8α-peroxide

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[1] Presented in part at the 31st Congrès Annuel de l'Association Canadienne-Française pour l'Avancement des Sciences, Quebec City, Canada, November, 1963. For Part XXV of this series, see P. Hill and D. Dvornik, Proc. Soc. Exp. Biol. Med. *122*, 1223 (1966).

[2] Abbreviations: 22,25-DAC (SC-11952), 20α-(2-dimethylaminoethyl)amino-5α-pregnan-3β-ol dihydrochloride; AY-9944, *trans*-1,4-*bis*(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride; triparanol (Mer-29), 1-[p-(β-diethylaminoethoxy)phenyl]-1-(p-tolyl)-2-(p-chlorophenyl) ethanol; 20,25-DAC (SC-12937), N-methyl-N-(3-dimethylamino) propyl-17β-aminoandrost-5-en-3β-ol; Δ^{7}-reductase, 7-dehydrocholesterol-Δ^{7}-reductase; Δ^{24}-reductase, 24-dehydrosterol-Δ^{24}-reductase; MVA, mevalonic acid; HMGCoA, hydroxymethylglutarylcoenzyme A; DBED, N,N'-dibenzylethylenediamine.

TABLE I

Effect of AY-9944, 22,25-DAC and Triparanol on the Conversion of 2-C^{14}-MVA and 26,27-C^{14}-Desmosterol to cholesterol by Rat Liver Homogenates[a]

Inhibitor	Final conc.	Mevalonate as precursor: Dpm in cholesterol[b]	Mevalonate as precursor: Inhibition %	Desmosterol as precursor: Dpm in cholesterol	Desmosterol as precursor: Inhibition %
None	—	37,492	—	88,287	—
AY-9944	1.0×10^{-5}M	27	100	92,714	0
	1.0×10^{-6}M	595	98	82,821	7
	1.0×10^{-7}M	36,716	7	—	—
22,25-DAC	1.0×10^{-5}M	60	100	3,625	96
	1.0×10^{-6}M	967	97	4,242	95
Triparanol	1.0×10^{-4}M	151	100	8,941	90
	1.0×10^{-5}M	11,703	69	26,408	70

[a]Values reported are averages from incubations in duplicate.

[b]Cholesterol added as carrier; isolated, and counted as 5,6-dibromocholestan-3β-ol (13) and calculated as dpm/mg of cholesterol.

(4,12,13). In the assay of Kandutsch, the enzymatic conversion of 7-dehydrocholesterol to cholesterol is determined by measuring the disappearance in the ultraviolet of the $\Delta^{5,7}$-diene system. Hence, the assay will register any reaction, other than the Δ^7-reductase involved in the formation of cholesterol, which causes disappearance of the $\Delta^{5,7}$-double bond, e.g. 5α,8α-peroxide formation. Assuming that peroxidation may occur, Kandutsch suggested to perform the assay with brain and kidney tissue under strict anaerobic conditions. It is pertinent to note that in the presence of AY-9944, the level of 7-dehydrocholesterol in the Kandutsch test remained unchanged, thus indicating that, with our experimental conditions, the disappearance of 7-dehydrocholesterol in control experiments was indeed due to the Δ^7-reductase activity of the liver homogenate.

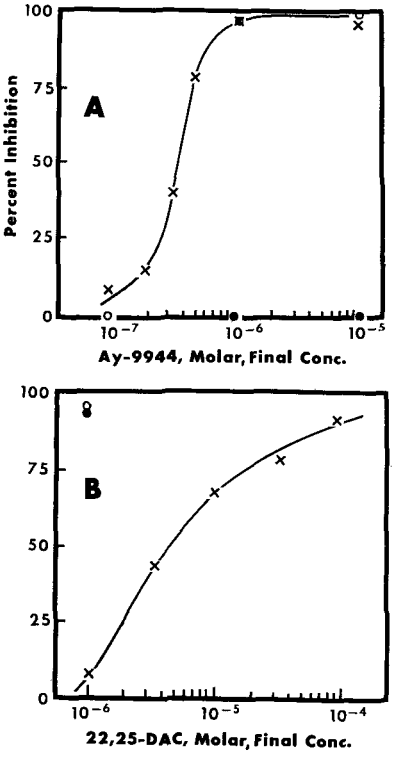

FIG. 1. The inhibition of 7-dehydrocholesterol-Δ^7-reductase by AY-9944 (A) and 22,25-DAC (B). Values for inhibition of cholesterol biosynthesis with MVA (o) and desmosterol (●) as substrates are also included.

RESULTS

The results obtained with 22,25-DAC, AY-9944 and triparanol are presented in Table I and Fig. 1A and B.

As reported earlier (4), AY-9944 is a potent inhibitor of the Δ^7-reductase and 50% inhibition is obtained with a final concentration of 4.0×10^{-7} M (cf. Fig. 1A). Similar data were also reported by Niemiro and Fumagalli (17). Within the limits of the experimental error, AY-9944 inhibits the incorporation of MVA into cholesterol to the same extent as it inhibits the Δ^7-reductase. At concentrations up to 1.0×10^{-4} M, AY-9944 had no effect on the conversion of desmosterol to cholesterol and thus behaves like a "pure" Δ^7-reductase inhibitor. Recently, three new types of Δ^7-reductase inhibitors were described (18) but their specificity regarding the Δ^{24}-reductase system was not reported.

22,25-DAC, on the other hand, suppressed the incorporation of MVA into cholesterol to the same extent as it inhibited the Δ^{24}-reductase: in both instances, and at a final concentration of 1.0×10^{-6} M, the inhibition was over 90%. At this concentration, 22,25-DAC had virtually no effect on the Δ^7-reductase system (Fig.

1B). However, at higher concentrations, 22,25-DAC affected the Δ^7-reductase: 50% inhibition was observed with 4.5×10^{-6} M, while at 1.0×10^{-4} M the inhibition exceeded 90%. Hence, 22,25-DAC behaved in vitro like a "mixed" inhibitor blocking both the Δ^7- and the Δ^{24}-sterol reductase systems.

With triparanol, the inhibition of MVA incorporation into cholesterol parallelled its block of the Δ^{24}-reductase (70% inhibition in both cases at 1.0×10^{-5} M). At the highest concentration tested (1.0×10^{-4} M), triparanol had no significant effect on the Δ^7-reductase.

DISCUSSION

Previous work in vivo (3,14,19) and in vitro (6) has indicated that the major site of inhibition of cholesterol biosynthesis by 22,25-DAC was at the level of the enzymatic reduction of the $\Delta^{24,25}$ double bond in the biogenetic precursors of cholesterol. The present study indicates that, in vitro, 22,25-DAC can also inhibit the Δ^7-reductase, another enzyme system involved in the biosynthesis of cholesterol (4). 20,25-DAC, a close analog of 22,25-DAC, has also been found to be a "mixed" inhibitor active on both the Δ^7- and Δ^{24}-reductase systems (17). As reported for 22,25-DAC in the present study, 20,25-DAC was also a more potent inhibitor of the 24-reductase system.

In spite of the fact that, in vitro, 22,25-DAC has one-tenth the activity of AY-9944 as a Δ^7-reductase inhibitor, in vivo, in tissues of rats treated orally with 22,25-DAC, we were able to detect only desmosterol (3). No "fast-acting" sterols[3] which would result from Δ^7-reductase inhibition (15) were detectable. Hence, in vivo, 22,25-DAC affected only the Δ^{24}-reductase system. Since, in vitro, nearly a 100 times higher concentration of 22,25-DAC is required to inhibit the Δ^7-reductase to the same extent as the Δ^{24}-reductase, it is unlikely that, in vivo, 22,25-DAC can reach levels required to maintain effective Δ^7-reductase inhibition.

Recently, Pottier has reported on a series of 22,25-diaza-19-nor-cholesta-1,3,5-triene-3-ol derivatives with the capacity to inhibit both the Δ^7- and Δ^{24}-reductase systems of the cholesterogenic pathway (24). The effect on the Δ^7-reductase was apparently variable and with some compounds could be seen only on prolonged treatment with high doses.

In our hands, at the highest concentration tested (1.0×10^{-4} M final) triparanol had no significant effect on the Δ^7-reductase. Other investigators (17,25) have been able to demonstrate a weak but definite in vitro inhibition of Δ^7-reductase at somewhat higher triparanol concentrations. Thus, 20,25-DAC, 22,25-DAC, the compounds described by Pottier and triparanol, respectively, form a class of cholesterol biosynthesis inhibitors which are quite active on the Δ^{24}-reductase and considerably less active on the Δ^7-reductase system. AY-9944, on the other hand, is a potent inhibitor of the Δ^7-reductase with virtually no effect on the Δ^{24}-reductase system.

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[3]The rates of reaction and the intensities of colors developed by different sterols in the Schoenheimer-Sperry-Liebermann-Burchard test (20) reflect the structure of the sterol. Thus, at room temperature, Δ^5-sterols reach maximal color intensity after 30-35 min. In contrast, Δ^7-, $\Delta^{5,7}$-sterols and their 5α,8α-peroxides (15) or compounds readily convertible into the former, produce a maximum almost immediately and are therefore termed "fast-acting" sterols (21-23).

Lysosomes and Essential Fatty Acid Deficiency

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ABSTRACT

The hydrolytic activity usually associated with lysosomes increased in the homogenates and subcellular fractions of rat liver as a result of essential fatty acid (EFA) deficiency. The proportion of the total (tissue homogenate) activity found in each subcellular fraction, however, was unchanged by EFA deficiency.

Lysosomes isolated from normal and EFA-deficient rat livers differed significantly in their stability to thermal and osmotic variations. This suggested that lysosomal membranes, like other membranes, were altered by EFA deficiency.

In spite of increased tissue-bound hydrolytic activity and altered lysosomal membranes, hydrolytic activity of the serum was not markedly changed in EFA deficiency. These minor changes in hydrolytic activity and in lysosomal membrane stability seemed insufficient to explain the general lesions of EFA deficiency.

INTRODUCTION

DESPITE MANY INVESTIGATIONS (1), a unified biochemical explanation for EFA deficiency symptoms has yet to appear. However, evidence is accumulating that at least some symptoms of the deficiency result from altered structure and permeability of biological membranes. In EFA deficiency, the skin usually undergoes extensive change (2-4) and becomes extremely permeable to water (5). With some species, susceptibility to respiratory and epidermal infections increases (6-9). Proteinuria, hematuria and urogenital degeneration may be observed (10-11). Capillaries of EFA-deficient rats become much more fragile and permeable than controls, even before the onset of secondary symptoms (12). EFA-deficient erythrocytes are more labile to osmotic lysis (5). There is abundant evidence that subcellular membranes too are altered in deficiency. EFA-deficient mitochondria, for example, are much less stable to isolation procedures and other physical stresses (14-18).

These findings are not entirely surprising, of course. It is well established that phospholipids, which play an important role in membrane structure, have a high EFA content (13,19,20) and are significantly altered in fatty acid composition by EFA deficiency (20-24). The general replacement of di- and tetraenoic acids with mono- and trienoic acids (16,21-23) could well be sufficient to affect the structural integrity of phospholipid-containing membranes.

It was interesting to speculate, therefore, that lysosomal membranes, in light of their phospholipid content (25), might undergo alterations in EFA deficiency. If so, the postulated digestive function of lysosomal particles (26) should also undergo alteration. The resulting digestion of functional cell components or lack of digestion of accumulating substances in the cell might then contribute to the secondary symptoms of the deficiency. An analogous correlation between lysosomal membrane instability and vitamin E deficiency symptoms has already been suggested (27).

The present experiments were designed to examine the possibility that lysosomal enzymes may play an important role in the symptomology of EFA deficiency. It will be shown that although the deficiency does alter some lysosomal characteristics, the changes do not appear sufficient to account for EFA deficiency symptoms.

EXPERIMENTAL PROCEDURE

Experimental Animals

Weanling, male albino rats (Holtzman Company, Madison, Wisconsin) were placed on casein-cerelose type diets, either fat-free or with corn oil replacing 5% (by weight) of the cerelose (28). The animals, individually housed in hanging wire cages and given food and water ad libitum, were maintained at least 12 weeks before use.

Methods

Ten per cent homogenates of kidney or liver were prepared in cold 0.25 M sucrose (0.001 M EDTA, pH 7.0) by "light" Potter-Elvejhem homogenization, and the classical subcellular fractions prepared essentially according to Hogeboom (29). However, each debris pellet

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was washed two times with 0.25 M sucrose, while mitochondrial and microsomal pellets were not washed. The debris and mitochondrial pellets were sedimented by 10-min centrifugations at $600 \times g$ and $8,000 \times g$, respectively, while 1 hr at $105,000 \times g$ (R_{avg}) produced the microsomal pellet.

To effect complete release of enzymes from lysosomal particles, each subcellular fraction and an aliquot of unfractionated homogenate were diluted to known volumes with 0.1% Triton x-100, a nonionic detergent. After enzyme assays, dilution factors were used to calculate the total enzyme activity contained in 10 ml of homogenate and in each complete fraction obtained from that 10 ml of homogenate. Subsequently, the nitrogen content of each homogenate was determined using the method of Johnson (30), except that sample digestion was carried out in 30-ml micro-Kjeldahl flasks. Then, the total enzyme activity of the tissue, as determined by assay of the homogenate, and the activity of each fraction were expressed as mμmoles product/mg tissue N/min. The proportion of activity in each fraction was calculated as percentage of the sum of the activities of the four fractions. The sum of the activities of the four fractions divided by the total activity of the unfractionated homogenate permitted calculation of "per cent yield."

For each membrane stability determination, mitochondrial fractions from 40 ml of liver homogenate or 30 ml of kidney homogenate were made to 10 ml in 0.7 M sucrose, in which lysosomes were reportedly most stable (31). As quickly as possible, equal aliquots of this suspension were diluted to 10 ml, yielding equal concentrations of lysosomes suspended in 0.08% Triton x-100 or in varying concentrations of sucrose, 0.1 M to 0.7 M, buffered with 0.01 M, pH 7.4 tris-(hydroxymethyl)-aminomethane. Incubation at 37C was initiated immediately after dilution, and at 1, 30 and 60 min after dilution, 3-ml aliquots were transferred to cold centrifuge tubes. Intact particles were rapidly sedimented at $20,000 \times g$ for 10 min, and the resulting sucrose supernatants and detergent suspensions were assayed for enzyme activity. The activity of each sucrose supernatant, times 100, was divided then by the activity of the detergent-treated suspension, which represented total enzyme release. This indicated what percentage of the total activity of the suspended lysosomes had been released by each osmotic and incubation treatment.

Enzyme Assays

All liver preparations were assayed for acid phosphatase activity with *p*-nitrophenyl phosphate as substrate. Kidney preparations were assayed for cathepsin activity (hemoglobin substrate) and, like serum, assayed also for acid phosphatase (β-glycerophosphate substrate) and aryl sulfatase (*p*-nitrocatechol sulfate substrate). For all assays substrate and enzyme concentrations and incubation times were adjusted to yield zero order kinetics with regard to substrate. Each sample involved duplicate determinations, as well as substrate and enzyme blanks.

Acid phosphatase (*p*-nitrophenyl phosphate), according to Lowery et al. (32), was measured in 1.0 ml total volume which was 0.05 M in succinate, pH 5.3, 0.0025 M in MgCl, 18.8 mM in *p*-nitrophenyl phosphate and 0.7 M in sucrose. Trichloroacetic acid (TCA) (8% w/v) was used for enzyme inactivation following 10 min incubation at 37C. The concentration of *p*-nitrophenol was measured by absorption at 410 mμ.

Acid phosphatase (β-glycerophosphate) was measured according to Gianetto and De Duve (33), with released phosphate measured according to Lowrey et al. (34). The assay flasks contained 1.0 ml total volume which was 0.05 M in acetate, pH 5.0 and 0.05 M in β-glycerophosphate. After 15 min incubation at 37C, TCA (0.3 M) was added to inactivate the enzyme. Phosphate was determined by absorption of the colored complex at 820 mμ.

Aryl sulfatase, measured according to Roy (35), was assayed in 1.0 ml total volume which was 0.05 M in acetate, pH 5.5 and 0.005 M in *p*-nitrocatechol sulfate. Phosphotungstic acid was used for inactivation after an incubation of 1 hr at 37C. Absorption at 520 mμ by the *p*-nitrocatechol-color reagent mixture indicated *p*-nitrocatechol concentration.

RESULTS AND DISCUSSION

As indicated in Table I, total acid phosphatase activity of the liver was increased in EFA deficiency. This increase of about 15% was highly significant—at the 0.005 level according to the F test. The largest absolute increase occurred in the microsomal fraction, but all fractions were proportionally increased.

As shown in Table II, means of duplicate assays for 3 hydrolytic enzymes of kidney also indicated increased activity in EFA deficiency, reflecting the same differences that exist between normal and EFA-deficient livers.

TABLE I

Subcellular Distribution of Acid Phosphatase Activity (p-nitrophenyl phosphate) in Normal and Essential Fatty Acid (EFA)-Deficient Rat liver

Fraction	Enzyme activity[a,b] Control	EFA-def't	Level of significance
	(mμmoles/mg tissue N/min)		(p)
Debris	142 (11)	182 (9)	.05
Mitochondrial	165 (6)	175 (12)	>.10
Microsomal	345 (12)	409 (22)	.05
Supernatant	125 (7)	134 (7)	>.10
Σ[c]	—	—	
	777 (32)	900 (45)	.10
Total[d]	792 (11)	904 (26)	.005

[a] Means of assays on 5 control and on 5 EFA-deficient liver preparations, 1 animal per preparation.

[b] Standard error of the mean in parentheses.

[c] Sum of the activities of the 4 fractions.

[d] Measured by assay of unfractionated homogenate.

These apparent differences in normal and EFA-deficient kidneys did not assume statistical significance, however.

As Table III shows, the proportions of acid phosphatase activity in corresponding normal and EFA-deficient liver fractions were not significantly different, in spite of significant differences in absolute activity of the fractions (Table I). In kidney fractions, likewise, the percentage distribution of acid phosphatase (Table III) and of aryl sulfatase and cathepsin activities (Table IV) appeared to be unchanged by EFA deficiency.

Table III illustrates a pronounced difference in the response of liver and kidney to homogenization and in the sedimenting characteristics of liver and kidney lysosomes. The control kidney microsomal fraction contained a smaller proportion of the acid phosphatase activity than did the control liver microsomal fraction ($p=0.005$). Control kidney debris and mitochondrial fractions, on the other hand, contained larger proportions of the total activity than corresponding fractions from normal liver ($p=0.005$ and 0.025, respectively). The proportions of activity in control liver and kidney supernatant fractions were not significantly different, however.

It is conceivable that a general leucocyte invasion of tissues could occur in the diseased state produced by EFA deficiency, and result in increased hydrolytic activity in the liver. But, the increased hydrolytic activity observed in EFA-deficient liver occurred without a change in percentage distribution of the activity in the subcellular fractions. Therefore, the increased hydrolytic activity could have resulted from leucocyte invasion only if leucocytes had sedimentation properties identical to those of liver lysosomes.

Three different "tracer" enzymes failed to indicate a uniform subcellular distribution of kidney lysosomes (Table III and IV). Nevertheless, the subcellular distribution of each "tracer" enzyme assayed was the same for normal and EFA-deficient preparations of liver or of kidney. Therefore, it appeared that the sedimenting characteristics of lysosomes from a given organ were essentially unchanged by EFA deficiency. Consequently, the same subcellular fraction, i.e., mitochondrial fraction, from control and EFA-deficient homogenates was used as a source of lysosomes for membrane stability studies.

From Fig. 1 it is seen that EFA-deficient liver lysosomes released less acid phosphatase activity than control lysosomes when suspended in 0.15 M sucrose. The difference

TABLE II

Subcellular Distribution of Aryl Sulfatase, Acid Phosphatase (β-glycerophosphate) and Cathepsin Activities in Normal and Essential Fatty Acid (EFA)-Deficient Rat Kidney

Fraction	Enzyme activity[a,b] Aryl sulfatase Control		EFA-def't		Acid phosphatase Control		EFA-def't		Cathepsin Control		EFA-def't	
	(mμmoles/mg tissue N/min)											
Debris	66	(4)	61	(11)	498	(21)	601	(188)	17	(1)	24	(5)
Mitochondrial	35	(1)	43	(14)	375	(58)	522	(14)	16	(1)	27	(5)
Microsomal	11	(0)	13	(4)	357	(44)	420	(42)	8	(1)	14	(4)
Supernatant	12	(1)	16	(8)	239	(19)	322	(96)	6	(1)	11	(3)
Σ[c]	—		—		—		—		—		—	
	124	(3)	133	(37)	1469	(140)	1865	(339)	47	(4)	76	(18)
Total[d]	105	(1)	129	(32)	1309	(11)	1779	(216)	49	(2)	79	(21)

[a] Means of assays on 2 control and on 2 EFA-deficient kidney preparations, 2 animals per preparation.

[b] Standard error of the mean in parentheses.

[c] Sum of the activities of the 4 fractions.

[d] Measured by assay of unfractionated homogenate.

TABLE III

Percentage Distribution of Acid Phosphatase Activity in Subcellular Fractions of Normal and Essential Fatty Acid (EFA)-Deficient Rat Liver (*p*-nitrophenyl phosphate) and Kidney (*β*-glycerophosphate)

Fraction	Liver[a,b]		Kidney[b,c]	
	Control	EFA-def't	Control	EFA-def't
	(%)			
Debris	18.3 (0.9)	20.2 (0.5)	34.0 (1.8)	32.2 (4.4)
Mitochondrial	21.2 (0.5)	19.5 (0.7)	25.5 (1.2)	28.0 (4.5)
Microsomal	44.4 (0.6)	45.4 (1.1)	24.3 (0.8)	22.5 (1.8)
Supernatant	16.1 (0.6)	14.9 (0.5)	16.2 (0.3)	17.3 (2.0)
Σ	100.0	100.0	100.0	100.0
% yield[d]	98.1 (3.8)	99.6 (5.6)	112.2 (9.7)	104.8 (6.5)

[a] Means of assays on 5 control and on 5 EFA-deficient liver preparations, 1 animal per preparation.

[b] Standard error of the mean in parentheses.

[c] Means of assays on 2 control and on 2 EFA-deficient kidney preparations, 2 animals per preparation.

[d] (Σ activity/homogenate activity) × 100%.

TABLE IV

Percentage Distribution of Aryl Sulfatase and Cathepsin Activities in Subcellular Fractions of Normal and Essential Fatty Acid (EFA)-Deficient Rat Kidney[a,b]

Fraction	Aryl sulfatase		Cathepsin	
	Control	EFA-def't	Control	EFA-def't
	(%)			
Debris	53.7 (1.8)	45.5 (4.8)	35.2 (0.9)	31.5 (0.4)
Mitochondrial	27.9 (1.5)	32.5 (1.8)	34.8 (0.4)	35.7 (1.4)
Microsomal	9.0 (0.5)	10.1 (0.1)	16.4 (1.0)	18.4 (0.6)
Supernatant	9.4 (0.3)	11.9 (3.0)	13.6 (0.4)	14.4 (1.1)
Σ	100.0	100.0	100.0	100.0
% yield[c]	118.2 (1.3)	103.2 (3.4)	96.3 (12.6)	96.7 (3.2)

[a] Means of assays on 2 control and on 2 EFA-deficient kidney preparations, 2 animals per preparation.

[b] Standard error of the mean in parentheses.

[c] (Σ activity/homogenate activity) × 100%.

became significant at the 0.025 level, however, only after 60 min incubation at 37C. In contrast, the EFA-deficient lysosomes in 0.45 M and 0.70 M sucrose released *more* activity and the differences *diminished* in significance with incubation time. The observed differences were significant (0.01 to 0.005 level) after 1 min in 0.45 M sucrose and after 1 and 30 min in 0.7 M sucrose. Possibly, lysosomal membranes become more permeable in EFA deficiency. Greater permeability would allow "leakage" of small endogenous molecules, making the EFA-deficient lysosomes less labile to osmotic lysis in a hypotonic medium. The experimental results, including the time-dependency of the differences, would support this hypothesis. On the other hand, the more rapid rate of release of enzymes from EFA-deficient lysosomes in a hypertonic medium suggests that EFA-defi-

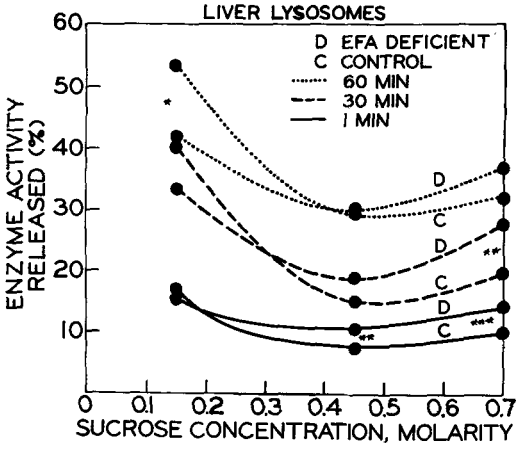

FIG. 1. Per cent acid phosphatase (*p*-nitrophenyl phosphate) activity released from normal and EFA-deficient liver lysosomes suspended in solutions of various sucrose concentrations and incubated at 37C. Data are averages from measurements on 6 control and 9 EFA-deficient preparations, 1 animal per preparation.

$*p = 0.025$; $**p = 0.010$; $***p = 0.005$.

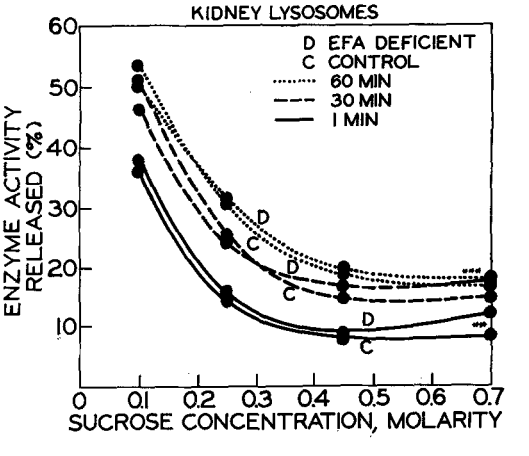

FIG. 2. Per cent of acid phosphatase (*β*-glycerophosphate) activity released from normal and EFA-deficient kidney lysosomes suspended in solutions of various sucrose concentrations and incubated at 37C. Data are averages of measurements on 5 control and 4 EFA-deficient preparations, 2 animals per preparation.

$**p = 0.010$; $***p = 0.005$.

TABLE V

Effect of Sucrose Concentration and 37C Incubation Time on Release of Aryl Sulfatase and Cathepsin Activities from Kidney Lysosomes of Normal and EFA-Deficient Rats[a,b]

Sucrose concentration	Incubation time (37C)	Aryl sulfatase		Cathepsin	
		Control	EFA-def't	Control	EFA-def't
0.10 M sucrose:	1 min	37 (5)	37 (5)	45 (4)	44 (5)
	30 min	56 (8)	55 (3)	62 (4)	68 (5)
	60 min	54 (4)	56 (2)	71 (5)	77 (8)
0.25 M sucrose:	1 min	19 (2)	20 (1)	18 (4)	7 (4)
	30 min	37 (2)	39 (3)	*33 (3)	*18 (2)
	60 min	46 (1)	46 (3)	44 (3)	34 (7)
0.45 M sucrose:	1 min	12 (2)	11 (1)	9 (4)	3 (3)
	30 min	21 (2)	24 (3)	*22 (4)	*8 (3)
	60 min	28 (2)	30 (3)	26 (4)	14 (5)
0.70 M sucrose:	1 min	13 (2)	15 (1)	17 (5)	9 (5)
	30 min	22 (3)	22 (3)	*28 (4)	*15 (3)
	60 min	24 (2)	26 (1)	28 (3)	19 (6)

[a] Averages of 5 control and 4 EFA-deficient preparations, 2 animals per preparation.

[b] Standard error of the mean in parentheses.

* Significance level, $p = < 0.05$.

cient membranes are also more fragile than normal membranes. Obviously, thermal effects of the incubation treatment, peroxidation, or other factors may have influenced the experimental results. Because of the complexity of the system, the only conclusion possible is that lysosomal membranes from EFA-deficient rat liver are significantly different from those isolated from normal rat liver.

As Fig. 2 shows, kidney lysosomes also tended to release less activity in a hypotonic system, and to release more in hypertonic suspension. These differences, obviously less marked than those from liver data, were significant in the 0.7 M sucrose suspensions. Table V indicates that the rates of aryl sulfatase release from kidney lysosomes showed no significant differences between normal and EFA-deficient lysosomal membranes. Significantly less cathepsin activity was released from EFA-deficient lysosomes, however, after 30-min suspension in 0.25 M, 0.45 M and 0.70 M sucrose.

EFA deficiency then seemed to result in: 1) a rather small but highly significant increase in hydrolytic activity levels in liver, and 2) small but ill-defined changes in stability of liver lysosomal membranes. Similar, but smaller and statistically insignificant differences were noted in normal and EFA-deficient kidneys and kidney lysosomes. If the small differences observed were to have physiological significance, one might expect a change in the steady-state level of free hydrolytic activity in the serum.

Serum acid phosphatase activity of 9 control and 7 EFA-deficient rats averaged 186(21) and 154(14) mμmoles/ml serum/min, respectively. (Standard error of the mean appears in parentheses.) This difference was not significant. Serum aryl sulfatase activity of 9 control and 8 EFA-deficient rats averaged 91(8) and 116(6) mμmoles/ml serum/min respectively. This difference was significant at the 0.05 level. Apparently then, in vivo levels of free hydrolytic activity were neither uniformly nor markedly altered by EFA deficiency, in spite of increased enzyme activity and altered lysosomal membrane stability in certain tissues.

In summary, it appears that lysosomal membranes, like other biological membranes, may be adversely affected by EFA deficiency. The magnitude of these stability differences seems insufficient to contribute significantly to the gross physiological symptoms of the deficiency, however. Further, lysosomal enzyme activity increases significantly in the liver and apparently tends to increase in the kidneys of EFA-deficient rats. This increase could, of course, be another symptom of EFA deficiency, rather than an initial, direct cause of secondary symptoms. We have not eliminated the possibility that an even greater local build up of lysosomes may be responsible for the tail necrosis or urogenital degeneration occasionally seen in EFA-deficient rats. Nonetheless, the data and the considerations discussed make it highly probable that lysosomes have no primary importance in producing the general physiological lesions of EFA deficiency.

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The Nature of the Stimulatory Role of the Supernatant Fraction on Triglyceride Synthesis by the α-Glycerophosphate Pathway[1]

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ABSTRACT

Evidence is presented as to the nature and mechanism of the stimulatory effect of the supernatant fraction on the biosynthesis of triglycerides via the α-glycerophosphate pathway in the intestinal mucosa. When microsomes are employed as the enzyme source, the major lipid formed from either labeled palmitic acid or L-α-glycerophosphate is phosphatidic acid and only a limited amount of triglyceride is synthesized. The addition of the supernatant fraction to microsomes results in a stimulation of triglyceride biosynthesis at the expense of phosphatidic acid. Employing the same microsomal fraction, the reaction sequence was followed step by step and the intermediates were isolated. The results suggest that the stimulatory role of the supernatant fraction can be attributed to the presence of L-α-phosphatidate phosphohydrolase (EC 3.1.3.4). The hydrolysis of the biosynthesized microsomal phosphatidic acid by the supernatant enzyme occurs at a faster rate than the hydrolysis of added phosphatidic acid prepared from egg lecithin. The initial acylation steps in the biosynthesis of triglycerides or phosphatidic acid via the glycerophosphate pathway occur only in the presence of fatty acid and the cofactors necessary for its activation. Under these conditions, fatty acyl-CoA will not substitute for the fatty acid activation system.

INTRODUCTION

THE BIOSYNTHESIS OF TRIGLYCERIDES via α-glycerophosphate pathway (1-3) by the intestinal mucosa was suggested several years ago (4). Additional evidence for the occurrence of this pathway in the intestine has been reported (5, 6). Clark and Hübscher (5) first presented evidence for a second pathway in the intestinal mucosa which utilizes monoglycerides for triglyceride synthesis. These results have been confirmed and extended by several laboratories (7-10). Both in vivo (11, 12) and in vitro (13) studies suggest that the monoglyceride pathway is the major pathway in this tissue. The enzymes catalyzing this reaction sequence are present in the microsomes (8, 14) and have been further purified from this fraction (15). The enzymes of the α-glycerophosphate pathway are also present in the microsomal fraction; however, in this case the presence of a 104,000 × *g* supernatant fraction markedly stimulates triglyceride synthesis (16, 17). Hübscher and associates have reported that the supernatant factor is heat sensitive and precipitated by ammonium sulfate.

In the reported investigation, several facets of this pathway are presented that suggest the nature of the stimulatory role of the supernatant fraction on triglyceride biosynthesis.

MATERIALS AND METHODS

^{14}C-L-α-glycerophosphate was synthesized by the procedure of Tzur et al. (18) and ^{14}C-palmityl-CoA and palmityl-CoA by the procedures previously described (14). L-α-glycerophosphate was obtained from the California Corporation for Biochemical Research, Los Angeles. A sample of lysophosphatidic acid was kindly supplied by W. E. M. Lands. Phosphatidic acid sodium salt, which was prepared enzymatically from egg lecithin was obtained from the Pierce Chemical Co., Rockford, Ill. The purity of the I-^{14}C-palmitic acid was 99% as judged by thin-layer chromatography (TLC). The subcellular fractionation was carried out employing a 20% homogenate of hamster intestinal mucosa via the procedure previously described (14). The conditions for incubation are given with the tables. The isolation of the lipids and their fractionation into classes were performed by the methods previously reported (14). The separation of phospholipids was carried out by a modification of the procedure described by Kuhn and

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Lynen (19). The Silica Gel G TLC plates were activated at 110C for 30 min. The aliquot of the lipid extract was then placed on the plate and developed in a solvent system consisting of diethyl ether; methanol; acetic acid (98:2:0.2, v/v). In this developing system, the mono-, di- and triglycerides, fatty acid and cholesterol esters migrate with or close to the solvent front and the phospholipids remain at the origin. Following the development in this system the plates were immediately placed in a polyethylene bag which was flushed with dry high-purity nitrogen until the odor of the solvents was not detectable. The plates were then developed in chloroform:methanol:30% methylamine (65:25:8, v/v) in lined tanks. The phospholipids were visualized by spraying with 0.2% 2′, 7′-dichlorofluorescein in ethanol and the radioactivity determined as previously described (20). When labeled palmitic acid was employed, the results were corrected for the trace amount of radioactivity present in the lipid fractions which was not L-α-glycerophosphate dependent. Inorganic phosphate was determined as described by Penniall (21).

RESULTS

Incubations were conducted under the conditions described in Table I employing I-^{14}C-palmitic acid or ^{14}C-L-α-glycerophosphate. A relatively high concentration of L-α-glycerophosphate was required for an appreciable synthesis of triglyceride which was α-glycerophosphate dependent. The labeled α-glycerophosphate which was not utilized in the biosynthesis was recovered unchanged. In some preliminary studies, in which varying concentrations of L-α-glycerophosphate were employed to determine the optimum concentration of this substrate, the K_m value was obtained. The value determined was 4.3×10^{-3} M which probably explains the rather high substrate requirements. In addition, the rather high K_m value may have certain physiological implications. As has been previously reported by Hübscher and co-workers (16, 17), it can be seen that the addition of the supernatant fraction markedly stimulated the synthesis of triglyceride from labeled palmitic acid (Flask 3) and the stimulatory factor was found to be heat-labile (Flask 4). The utilization of labeled L-α-glycerophosphate for triglyceride biosynthesis and the stimulatory effect of the supernatant fraction is also shown in this table (Flasks 5 and 6). It was observed that in addition to triglyceride synthesis, a considerable amount of activity failed to migrate in the developing solvent system (n-hexane: diethyl ether: methanol: acetic acid, 90:21:3:2, v/v) and remained at the origin. This polar lipid was present when either labeled palmitic acid or labeled L-α-glycerophosphate was the substrate. When this lipid was further fractionated by the chromatographic procedure for phospholipids described in the methods section, 80 to 90% of its activity migrated with authentic phosphatidic acid and the remaining with lysophosphatidic acid. Only trace amounts of radioactivity were found in sphingomyelin, phosphatidyl-choline and phosphatidyl-ethanolamine. The polar lipid is therefor referred to throughout the text as phosphatidic acid. As can be seen, when microsomes were employed, the major lipid synthesized from labeled palmitic acid or L-α-glycerophosphate was phosphatidic acid. The addition of the supernatant fraction resulted in a decrease in the activity of this lipid fraction. Since, under the condition of these experiments, only trace amounts of lipids such as 1, 2-diglycerides were found, the total lipid synthesized from either labeled substrate was obtained by the addition of the mμmoles of the substrates present in phosphatidic acid and triglyceride (Column 3). With the exception of the supernatant fraction (Flask 2), it can be seen that the total lipid synthesis for each of the labeled substrates, with the varying cellular fractions, was approximately the same. Of more fundamental interest was the ob-

TABLE I

Lipid Synthesis by the α-Glycerophosphate Pathway

Contents	mμmoles of I-^{14}C Palmitic acid or ^{14}C-L-α-glycerophosphate incorporation		
	Triglyceride	Polar lipid	Total lipid synthesized
Experiment I			
1. M	252	1276	1528
2. S	46	0	46
3. M + S	1022	492	1514
4. M + Boiled S	275	1104	1379
Experiment II			
5. M	105	792	897
6. M + S	546	366	912

Each flask conained KF (25 μmoles), $MgCl_2$ (10 μmoles), GSH (25 μmoles), CoA (0.5 μmoles), ATP (30 μmoles), palmitic acid (2 μmoles), L-α-glycerophosphate (20 μmoles), 0.5 ml of 0.5 M Tris Maleate buffer pH 7.0, microsome (M) and/or supernatant (S) corresponding to 1 ml of a 20% homogenate in a final volume of 2.5 ml. The homogenate was made using 0.01 M Tris Maleate buffer (pH 7.0) containing 0.278 M mannitol. Incubation was carried out at 37 C in a Dubnoff shaker for 1 hour. Reaction was stopped by adding 5 ml of chloroform-methanol (2:1, v/v). Flasks 1-4 contained I-^{14}C-palmitic acid and Flasks 5 and 6, ^{14}C-L-α-Glycerophosphate.

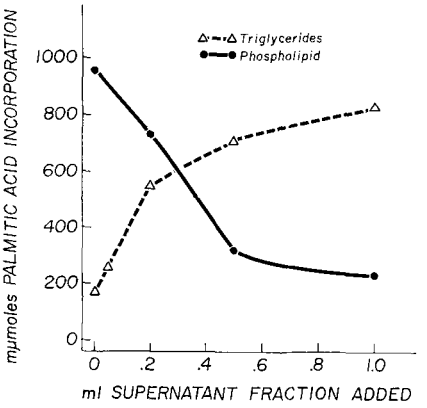

FIG. 1. Effect of addition of the supernatant fraction on the lipid synthesis by the α-glycerophosphate pathway. For details see the text.

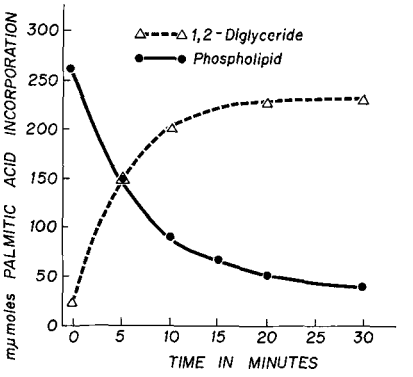

FIG. 2. Effect of addition of the supernatant fraction on the biosynthesized microsomal phosphatidic acid. For details see the text.

servation that the decrease in the phosphatidic acid concentration was paralleled by an increase in the triglyceride synthesis. For example, the addition of the supernatant fraction to the microsomes resulted in a decrease of 784 mμmoles of palmitate and 426 mμmoles of L-α-glycerophosphate incorporation into phosphatidic acid, and an increase of 770 and 441 mμmoles, respectively, of these substrates into triglycerides. These observations suggested that the stimulatory effect of the supernatant fraction on triglyceride biosynthesis is closely associated with the disappearance of phosphatidic acid.

In an effort to substantiate this suggestion, a series of incubations were performed under the same conditions given in Table I in which microsomes were incubated in the presence of increasing concentrations of the supernatant fraction. The results of this experiment are given in Fig. 1. A direct relationship of the stimulatory effect on triglyceride biosynthesis and the disappearance of phosphatidic acid was observed in the presence of a range of concentrations of the supernatant fraction.

In order to obtain further evidence for the reaction sequence as related to the microsomal and supernatant fractions, the following experiment was performed. The microsomal fraction was incubated for 30 min with I-^{14}C-palmitic acid under similar conditions as described in Table I. Following the incubation, the microsomes were isolated by centrifugation at 104,000 × *g* for 30 min and suspended in 0.154 M KCl. An equivalent of the supernatant fraction was added to the reisolated microsomes and an aliquot was immediately removed ("0" time). The incubation was carried out for 30 min and aliquots were removed for lipid analysis every 5 min. The results of this study are given in Fig. 2. As can be seen, throughout the 30-min period, a decrease in phosphatidic acid was observed which was paralleled by an increase in the formation of labeled 1, 2-diglyceride. In two control vessels in which the supernatant fraction was replaced by either Tris-Maleate buffer pH 7.0, or heat inactivated supernatant, only a trace amount of phosphatidic acid was converted into 1, 2-diglyceride. These results strongly suggest that at least one function of the supernatant fluid is the hydrolytic cleavage of the microsomal phosphatidic acid to form 1, 2-diglyceride.

The question as to whether the resulting diglycerides could serve as a precursor for triglyceride biosynthesis and whether the reaction sequence could be followed in a stepwise manner throughout the entire sequence was next investigated (Table II). The results are expressed in terms of similar aliquots. Incubation was carried out as described for Flask 1, Table I, except that twice the amount of microsomes was used and the time of incubation was 5 min. The microsomal bound phosphatidic acid was isolated by centrifugation and suspended in 0.154 M KCl. An aliquot was removed at this stage for lipid analysis (Flask 1). Three identical aliquots of preincubated microsomes were transferred into 3 flasks (Flasks 2, 3, and 4). To these flasks were added 0.5 M Tris-Maleate buffer pH 7.0, heat inactivated 104,000 × *g* supernatant fraction and 104,000 × *g* fresh supernatant fraction, respectively. The three flasks were then incubated at 37C for 10 min and

the microsomes reisolated from each flask, suspended in 0.154 M KCl and aliquots removed for analysis. As was previously observed, no appreciable hydrolysis occurred under the incubation conditions of Flasks 2 and 3. However, an appreciable hydrolysis occurred when the active supernatant fraction was present (Flask 4). Each of the remaining microsomal suspensions was divided into two equal portions and further incubated for 30 min at 37C with $MgCl_2$ (10 μmoles), GSH (25 μmoles), KF (25 μmoles) and 1 ml of 0.5 M Tris-Maleate buffer pH 7.0, and either 500 mμmoles of palmityl-CoA (Flasks 2a, 3a and 4a) or 500 mμmoles palmitic acid plus ATP (30 μmoles) and CoA (0.5 μmoles) in a final volume of 2.5 ml (Flasks 2b, 3b, and 4b). As can be seen from Table II, a marked stimulation of triglyceride biosynthesis occurred only in Flasks 4a and 4b. The increase in the synthesis of triglyceride during this incubation was 168 and 200 mμmoles with palmityl-CoA and palmitic acid, respectively. The decrease in the diglyceride fraction in these flasks was 161 and 193 mμmoles, respectively. Thus, an excellent agreement between the synthesis of triglycerides from the corresponding 1, 2-diglycerides was observed. It should be noted that both palmitic acid plus the activating system and palmityl-CoA can serve as substrates for the acylation of 1, 2-diglycerides to form triglycerides. The microsomal phosphatidic acid which had not been exposed to the active supernatant fraction and therefore not converted into 1, 2-diglycerides did not yield an appreciable synthesis of triglycerides. These results demonstrate that the synthesis of triglycerides occurs in the same microsomal fraction, provided the synthesized phosphatidic acid can be enzymatically converted to 1, 2-diglyceride. The enzyme present in the supernatant fraction for this hydrolysis is presumed to be L-α-phosphatidate phosphohydrolase (EC 3.1.-3.4).

Earlier studies of L-α-phosphatidate phosphohydrolase revealed its predominant presence in the microsomal fraction (22, 23) and hence this microsomal enzyme might be expected to hydrolyze the biosynthesized phosphatidic acid also in this fraction. However, the reported observations suggesting the presence of this hydrolase in the supernatant fraction led us to reexamine this problem. The activity of phosphohydrolase in the supernatant and microsomal fractions was determined by two procedures. In the first case, phosphatidic acid (prepared from egg lecithin) was added to

TABLE II

Intermediates of Triglyceride Biosynthesis by the α-Glycerophosphate Pathway

Flask contents	mμmoles of 1-^{14}C Palmitate in		
	Phosphatidic acid	1-2,DG	TG
1. Preincubated microsomes	300	5	45
2. Preincubated microsomes+buffer	278	22	43
(a) + palmityl-CoA	267	16	55
(b) + palmitic acid + CoA + ATP	266	5	81
3. Preincubated microsomes + heat inactivated supernate	271	18	45
(a) + palmityl CoA	269	12	62
(b) + palmitic acid + CoA + ATP	260	10	74
4. Preincubated microsomes+supernate	74	223	38
(a) + palmityl-CoA	74	62	206
(b) + palmitic acid + CoA + ATP	69	29	238

Conditions of incubation are described in text.

the fractions and the released P_i determined. Since the presence of mannitol interfered with the P_i determination, the subcellular fractionation was carried out in 0.25 M sucrose containing 0.001 M EDTA. The supernatant fraction thus obtained was identical to the mannitol system on the stimulatory effect of triglyceride synthesis. The substrate employed in the second assay procedure was bound biosynthesized phosphatidic acid. This substrate was prepared by incubating microsomes with ^{14}C-palmitic acid and L-α-glycerophosphate for 30 min. At the completion of the incubation the proteins were heat inactivated by boiling for 10 min at 100C. An aliquot was removed and the distribution of radioactivity in the various compounds determined by the method previously described. Aliquots containing 500 mμmoles of labeled phosphatidic acid were placed in each of several incubation vessels. The supernatant or microsomal fraction corresponding to 0.5 ml of a 20% homogenate was added and the flasks incubated for 1 hr at 37C. At the completion of the second incubation, the lipids were extracted and fractionated as described. The activity of the L-α-phosphatidate phosphohydrolase was determined by 1, 2-diglyceride formation. This assay procedure was necessary when the bound substrate was employed since, high protein concentrations have been shown to interfere with the determination of P_i. Although heat-inactivated bound phosphatidic acid was employed in the reported results, similar results were obtained when microsomal bound phosphatidic acid was employed as the substrate without heat inactivation. No release of P_i or 1, 2-diglyceride formation was observed in the control vessels in which either heat denatured microsomal or supernatant fractions was employed. When phosphatidic acid pre-

pared from egg lecithin was employed as the substrate, the hydrolysis was 42.4% for microsomal fraction and 8.0% for the supernatant fraction, thus confirming the earlier observations carried out under similar conditions that this enzyme was primarily associated with microsomes (22, 23). However, when the bound microsomal phosphatidic acid was utilized as the substrate the opposite relationship was observed in that only a 3.9% hydrolysis was observed when microsomes were employed compared to a phosphorylytic cleavage of 33.8% as catalyzed by the supernatant fraction. The explanation for the apparent difference in the relative rates of hydrolysis of microsomal bound biosynthesized phosphatidic acid and phosphatidic acid produced from egg lecithin is not clear. Several possible explanations such as their difference in fatty acid composition, the change in substrate reactivity by binding to a protein, and the effect on the activity of this enzyme by the formation of phosphatidic acid might be offered as possible suggestions for the different reactivity toward these two substrates. Experiments are now in progress in an attempt to resolve this question.

In addition to the properties of the supernatant factor already described, it was observed that this component is not retained by Sephadex G-100, suggesting a molecular weight in excess of 100,000. The factor is also –SH sensitive since exposure to N-Ethylmaleimide (0.4×10^{-3} M) followed by neutralization with an excess of 2-mercaptoethanol results in an 80% loss in its stimulatory effect on triglyceride biosynthesis. The requirement for –SH groups for phosphohydrolase activity has been reported by Coleman and Hübscher (23).

It has been shown that incubation mixtures containing both the supernatant and microsomal fractions are capable of synthesizing appreciable quantities of triglycerides from L-α-glycerophosphate and palmitic acid. Although it has been demonstrated that palmityl-CoA can substitute for the fatty acid activation system in the synthesis of triglycerides from 1, 2-diglycerides (Table II), early in the course of our studies, it was observed that palmityl-CoA cannot replace the fatty acid activation system in the acylation of L-α-glycerophosphate. The results which led to such conclusions are given in Table III. All the flasks contained microsomal and supernatant fractions corresponding to 1 ml of a 20% homogenate. The complete system (Flask 1) contained labeled 1-^{14}C-palmitic acid plus all the cofactors necessary for its activation and utilization for glyceride biosynthesis via the α-glycerophosphate pathway, as is described in Table I. As observed earlier, under these conditions, the incorporation of palmitate into triglycerides was considerable (906 mμmoles). When ^{14}C-palmityl-CoA was substituted for palmitic acid+ATP+CoA, no synthesis of triglycerides occurred (Flask 2). Although in the reported results a similar amount of palmityl-CoA was substituted for the palmitic acid, when palmityl-CoA was employed over a range of 50 to 2000 mμmoles no evidence was obtained for palmityl-CoA utilization. As previously mentioned when 500 mμmoles of palmityl-CoA was employed under conditions in which 1, 2-diglyceride was the acyl acceptor, triglyceride synthesis occurred (Table II). However, when this same concentration of palmityl-CoA was present with α-glycerophosphate no synthesis was detected. In addition, it was observed that the presence of unlabeled palmityl-CoA in a 1:1 ratio with labeled palmitic acid completely inhibited the utilization of the latter (Flask 3). When the effect of varying amounts of palmityl-CoA on palmitic acid utilization was investigated, the per cent of inhibition on palmitic acid utilization was 100, 78.4 and 24.7, respectively, when 2, 0.5 and 0.2 μmoles of palmityl-CoA were present with 2 μmoles of 1-^{14}C-palmitic acid (Flasks 3, 4 and 5). Furthermore, the addition of crystalline bovine serum albumin, heat-denatured microsomal protein, or the prior binding of palmityl-CoA by these proteins over a range of concentrations resulted in a similar inhibition as that observed when palmityl-CoA was directly added. No inhibitory effect was noted when acetyl-CoA or increasing concentrations of free CoA were present.

The observation that palmityl-CoA is not utilized for glyceride biosynthesis led us to reexamine the question as to whether there was

TABLE III

Effect of Palmityl-CoA on the α-Glycerophosphate Pathway

Flask contents	mμmoles of 1-^{14}C-palmitate incorporation into triglycerides
1. [a]Palmitic acid (2 μm) + ATP + CoA	906
2. [a]Palmityl-CoA (2 μm)	0
3. [a]Palmitic acid + ATP + CoA + palmityl-CoA (2.0 μm)	0
4. [a]Palmitic acid + ATP + CoA + palmityl-CoA (0.5 μm)	196
5. [a]Palmitic acid + ATP + CoA + palmityl-CoA (0.2 μm)	685

[a]Denotes tbe labeled substrate added.
Condition for incubation same as is given in Table I.

an absolute CoA requirement. This is difficult to ascertain since the endogenous contribution of CoA may be adequate for its catalytic function. In order to circumvent this problem, the supernatant fraction was passed through Sephadex G-50 to remove the endogenous free CoA (24) and employed along with microsomes to study glyceride synthesis in the presence and absence of CoA. In the presence of CoA, 480 mμmoles of palmitic acid were incorporated into triglycerides and when CoA was omitted, no synthesis of triglyceride occurred under these conditions. Similarly, when ATP was omitted no synthesis was observed.

DISCUSSION

The first indication that several subcellular fractions were required for maximal higher glyceride synthesis by the α-glycerophosphate pathway was provided by Stein et al. (2). More recently, Hübscher and colleagues (16, 17) have materially advanced our understanding of the factors involved in this stimulation by demonstrating that the addition of the supernatant fraction to microsomes resulted in a multifold increase in triglyceride biosynthesis and provided evidence for the protein nature of this factor by demonstrating its susceptibility to heat inactivation and $(NH_4)_2SO_4$ precipitation. In the reported investigation these observations have been further extended and an explanation for the observed stimulatory effect is presented. The primary synthesis of a polar lipid from either labeled L-α-glycerophosphate or palmitic acid has been demonstrated when microsomes alone were employed as the enzyme source. The identity of this fraction as phosphatidic acid has been established by TLC, and by its conversion to 1, 2-diglyceride which in turn can serve as a precursor of triglycerides.

Therefore, the reported stimulatory effect of the supernatant fraction on triglyceride synthesis has been localized at the biosynthetic steps following phosphatidic acid formation since the incubation in the absence of the supernatant fraction results in the accumulation of phosphatidic acid. Furthermore, the total incorporation of either substrate under the stimulated or unstimulated conditions is almost of an identical level and only varies in their relative distribution between phosphatidic acid and triglycerides. Also, a direct relationship between these two compounds has been documented by the demonstration that the increase in triglycerides by the addition of the supernatant fraction to microsomes, is paralleled by a decrease in phosphatidic acid. This conclusion is further supported by the isolation of the individual intermediates from the same microsomal fraction (Table II). From the presented evidence, it would appear that the intermediates are synthesized and utilized on the microsomes and the function of the supernatant fraction is to supply the enzyme L-α-phosphatidate phosphohydrolase (EC 3.1.-3.4) for the conversion of the microsomal phosphatidic acid to 1, 2-diglyceride. It has been reported (22, 23) that microsomes contain a significant quantity of this enzyme by assaying it employing phosphatidic acid and measuring the release of inorganic phosphate. Similar results were obtained when phosphatidic acid produced from egg lecithin was employed. However, when the biosynthesized microsomal bound phosphatidic acid was utilized as the substrate, the phosphohydrolase activity of the supernatant fraction was of a higher order of magnitude than that of the microsomes.

The presented kinetic data provide an explanation for the observed high substrate requirements for L-α-glycerophosphate and the reported preferential synthesis of triglycerides by the monoglyceride pathway. When the glyceride synthesis was studied under identical conditions, using either L-α-glycerophosphate or 2-monopalmitin, the K_m values obtained were 4.3×10^{-3} M and 6.8×10^{-4} M respectively. Therefore, on the basis of these values one would predict that the monoglyceride pathway would be the preferred sequence of the biosynthesis of triglycerides as observed in both *in vivo* and *in vitro* studies (11-13).

The failure of palmityl-CoA to replace palmitic acid plus its activation system in the initial acylation step of L-α-glycerophosphate is perplexing. As can be seen from Table II, this derivative is a satisfactory substrate for the acylation of diglycerides. Several recent publications have reported that fatty acid-CoA derivatives are inhibitory to a number of enzyme systems presumably due to their detergent properties (25, 26). The susceptibility of the α-glycerophosphate pathway to detergents such as the "Tweens" has already been documented. This effect was thought to be localized at the step between phosphatidic acid and diglyceride formation (7, 27). The reported results indicate that in addition, palmityl-CoA is not utilized even for the acylation steps prior to phosphatidic acid formation. Furthermore, palmityl-CoA inhibits the utilization of free fatty acid. The attempts to reverse this inhibitory effect by the addition of inert

proteins and the failure to duplicate the inhibition by acetyl-CoA might suggest that the mode of action of palmityl-CoA may be somewhat specific. The failure of palmityl-CoA to serve as a substrate cannot be explained on the basis of its hydrolysis by palmityl-CoA hydrolase (EC 3.1.2.2) since the release of CoA was determined by the use of Ellman's reagent (28) and the results suggest that significant amount of palmityl-CoA is available for lipid synthesis during the entire incubation. Recently, Goldfine has suggested the participation of acyl carrier protein in the acylation of α-glycerophosphate in the biosynthesis of phospholipid by enzymes from *Clostridium butyricum* (29). Whether a similar intermediate is involved in the reported reactions awaits further investigations.

ADDENDUM

Further evidence to support the conclusion that the stimulatory effect of the supernatant fraction is due to the presence of L-α-phosphatidate phosphohydrolase was obtained from studies on the purification of this factor. The activities for (a) the increased formation of triglyceride from α-glycerophosphate, (b) the formation of 1-2, diglyceride from microsomal bound phosphatidic acid, and (c) the formation of triglyceride from microsomal bound phosphatidic acid in the presence of ATP, CoA and fatty acids were all quantitatively recovered in the pH 5 fraction separated from the supernatant. Furthermore, the increase in the specific activity for the three assay procedures was 2.9, 2.7 and 3.1, respectively, suggesting that these are intimately related. Several protein fractionation procedures were also performed in an attempt to purify the supernatant factor which resulted in a loss in these activities. In this case the phosphohydrolase and the triglyceride stimulatory activities were simultaneously destroyed. The parallel increase in specific activity, as well as the simultaneous loss of these activities would further support the conclusions of this paper that the major role of supernatant fraction in the stimulation of triglyceride biosynthesis is to provide the enzyme L-α-phosphatidate phosphohydrolase.

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Interaction of Calcium Ions with Lecithin and Sphingomyelin Monolayers

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ABSTRACT

Dipalmitoyl lecithin and sphingomyelin monolayers have similar limiting areas, whereas their surface potentials are strikingly different. The double bond at the 4–5 position in sphingomyelin acts as an induced dipole in relation to the surface potentials. This was confirmed by the surface potential of hydrogenated sphingomyelin. The binding of calcium to lecithin and sphingomyelin monolayers resulted in an increase in surface potential. This increase was greater for the dipalmitoyl lecithin monolayer as compared to that for sphingomyelin. It is concluded that the binding of calcium ions to springomyelin monolayers is significantly reduced by the presence of the hydroxyl group at the 3-carbon position of the molecule.

INTRODUCTION

LECITHIN AND SPHINGOMYELIN are important components of biomembranes. It was shown by de Gier and Van Deenen (1) that for several animal species, the lecithin content of erythrocyte membrane decreases as the sphingomyelin content increases. This counterbalancing effect between lecithin and sphingomyelin which also influences the permeability of erythrocyte membrane (2,3), led us to investigate the surface properties of lecithin and sphingomyelin monolayers. This paper reports the interaction of calcium with the monolayers of dipalmitoyl lecithin, sphingomyelin and hydrogenated sphingomyelin.

EXPERIMENTAL

Materials

L-α-dipalmitoyl lecithin was purchased from Mann Research Laboratories (N.Y.). Beef heart sphingomyelin was supplied by Sylvana Chemical Company (Millburn, N. J.). Both samples showed single spots on the thin-layer chromatography (TLC) plate with the solvent system chloroform-methanol-water (80:35:5 v/v/v). Table I shows the fatty acid composition of the sphingomyelin, analyzed by gas-liquid chromatography by courtesy of the laboratory of E. H. Ahrens, Jr. (Rockefeller University, New York). Lipid solutions were prepared in hexane-methanol-chloroform (3:1:1 v/v/v). Hexane was found necessary for the proper spreading of monolayers. Inorganic chemicals of reagent grade and twice-distilled water were used in all experiments.

Surface Pressure and Surface Potential Measurements

The method of measuring surface pressures by a modified Wilhelmy plate, and surface potentials by a radioactive electrode has been described previously (4). The surface measurements were taken on subsolutions of 0.02 M NaCl and 0.01 M $CaCl_2$ at pH 5.6, and 25C. The molecular weights of dipalmitoyl lecithin and sphingomyelin, calculated from the fatty acid composition, are 752 and 846, respectively.

State of Monolayers

The state of a monolayer is generally determined by sprinkling a small amount of talc on the monolayer and then gently blowing air at the talc particles by means of a dropper. If the talc moves freely under air-stream, the monolayer is in the liquid state. If the talc moves very little or not at all, the monolayer is in the gel or solid state, respectively. The principle underlying these observations is that molecules in solid monolayers do not move past one another, in the gel state they move very little, and in the liquid state they move freely. Although this method is less quantitative than surface viscosity measurements to determine the state of monolayers, it is reproducible within ± 1 dynes/cm in determining changes in the state of monolayers.

RESULTS AND DISCUSSION

Surface Pressure-Area Curves

Figure 1 shows the surface pressure-area curves of dipalmitoyl lecithin, sphingomyelin and hydrogenated sphingomyelin on subsolutions of 0.02 M NaCl or 0.01 M $CaCl_2$ at pH 5.6 and 25C. The limiting area of sphingomyelin (42–43 A^2/molecule) is the same as

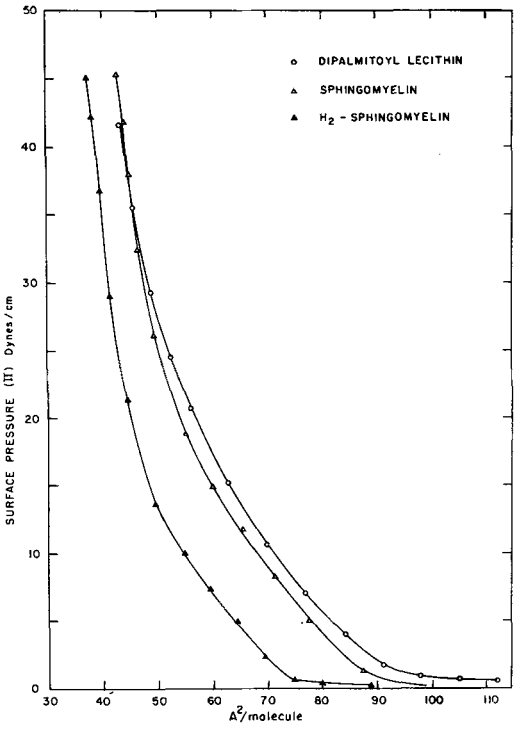

FIG. 1. Surface pressure-area curves of dipalmitoyl lecithin, sphingomyelin, and hydrogenated sphingomyelin on 0.02 M NaCl or 0.01 M $CaCl_2$ subsolutions, pH 5.6 at 25C.

that of dipalmitoyl lecithin, because sphingomyelin predominantly consists of saturated hydrocarbon chains (except for the double bond in the 4–5 carbon position) (5,6). The presence of C_{22}, C_{23} and C_{24} alkyl chains in sphingomyelin (Table I) contributes to the formation of a condensed monolayer due to the high Van der Waal interaction between hydrocarbon chains. The limiting area of sphingomyelin also agrees with that reported by Turner and Watson (7). It is interesting to note that the hydroxyl group in the polar part of sphingomyelin does not increase the limiting area.

TABLE I
Fatty Acid Composition of Sphingomyelin

Fatty acid[a]	Mole %
16:0	13.3
18:0	25.4
18:1	2.1
20:0	tr.
22:0	8.7
23:0	10.1
24:0	19.0
24:1	21.3

[a] Number of carbon atoms: number of double bonds.

Upon hydrogenation, the sphingomyelin monolayer shows a limiting area of 38 A^2/molecule. This reduction in limiting area is caused by the hydrogenation of the double bonds in hydrocarbon chains of the molecule, including the double bond in 4–5 position of sphingomyelin.

In contrast to soap monolayers (8–10), the surface pressure-area curves of lecithin and sphingomyelin are not influenced by the presence of calcium in the subsolution. It is emphasized here that the surface pressure-area curves do not indicate surface rheology, which was studied by the mobility of sprinkled talc particles. The dipalmitoyl lecithin monolayers on subsolutions of 0.02 M NaCl are in the liquid state up to a surface pressure of 35 dynes/cm, in the gel state from 35 to 40 dynes/cm, and in the solid state above 40 dynes/cm. These surface pressure values are reproducible within $\pm$ 1 dynes/cm. The presence of calcium (0.01 M) in the subsolution causes solidification of the lecithin monolayers at a lower surface pressure (30–33) dynes/cm). Sphingomyelin monolayers are in the liquid state up to the surface pressure of 40 dynes/cm on subsolutions of 0.02 M NaCl or 0.01 M $CaCl_2$. This difference in the state of lecithin and sphingomyelin monolayers is presumably due to the presence of a hydroxyl group on the 3-carbon position of sphingomyelin, which would increase the polar character of the sphingomyelin molecule, and influence the surface rheology of the monolayer. Hydrogenation of sphingomyelin did not significantly influence the state of sphingomyelin monolayers.

It is desirable to explain the significance of three parameters used in this paper, namely surface pressure, surface potential and surface rheology in relation to surface properties of the molecules in the film. The surface pressure-area curve indicates the compressibility of the material in the monolayer and does not provide any information regarding surface rheology of the compound, e.g., cholesterol (11) and dicetyl phosphate (4) both have similar surface pressure-area curves, although their surface rheology is strikingly different. Cholesterol forms liquid whereas dicetyl phosphate forms solid monolayers. In turn, the compressibility of a monolayer depends upon the ionic nature of the polar group, chain length, unsaturation and other steric factors of the molecules in the film. Surface rheology, which is a measure of the ability of molecules to move

past one another, is determined by three factors: firstly, the cohesive force between hydrocarbon chains of the molecules; secondly, the interaction between polar groups of the molecules; thirdly, the interaction of the molecules with the sub-solution (and/or with the substances present in the subsolution, e.g., metal ions, proteins, soluble surfactants, etc.). Changes in any of these factors would influence the rheology of monolayers (12,13).

In order to form a monolayer, it is absolutely essential that the molecules possess a nonpolar hydrocarbon chain as well as a polar (ionic or nonionic) group. The polar group of the molecule has a resultant dipole either due to *partial ionic charges* on atoms (e.g., $-O(\delta-)-H(\delta+)$ in alkyl alcohols) or due to a full ionic charge (e.g., $-COO^-Na^+$ in soap monolayers). Thus, the lattice composed of these dipoles can be mathematically treated as a parallel plate condenser. The potential across this condenser is related to surface charge and in turn to the individual dipole in the lattice (14). It should be emphasized that the relationship between surface potential and resultant dipole of the individual molecule is derived by using the parallel plate condenser model as a close approximation and this should not be thought of as an *exact* description of the electrical structure of the monolayer. Thus in general, surface potential is an indication of the electrical (or dipole) properties of the molecules in the monolayer.

Surface Potential-Area Curves

Figure 2 shows the surface potential-area curves of dipalmitoyl lecithin, sphingomyelin, and hydrogenated sphingomyelin on subsolutions of 0.02 M NaCl and 0.01 M $CaCl_2$. The surface potential, $\triangle V$, is related to the vertical component (μ) of the total dipole moment of the molecule and to the number of molecules per square centimeter in the film (15). The component dipoles of lecithin and plasmalogen in relation to their surface potentials have been reported previously (4). As in the case of plasmalogen, the presence of a double bond in sphingomyelin also acts as an induced dipole which significantly influences the total surface dipole and consequently the surface potential. It was shown by Hughes and Rideal (16) that in $\triangle^2$-octadecenoic acid, in which a double bond is vicinal to the carboxyl group, the surface dipole μ is twice that of its isomer oleic acid. Here the double bond situated immediately above the polar group possesses an induced dipole of the strength

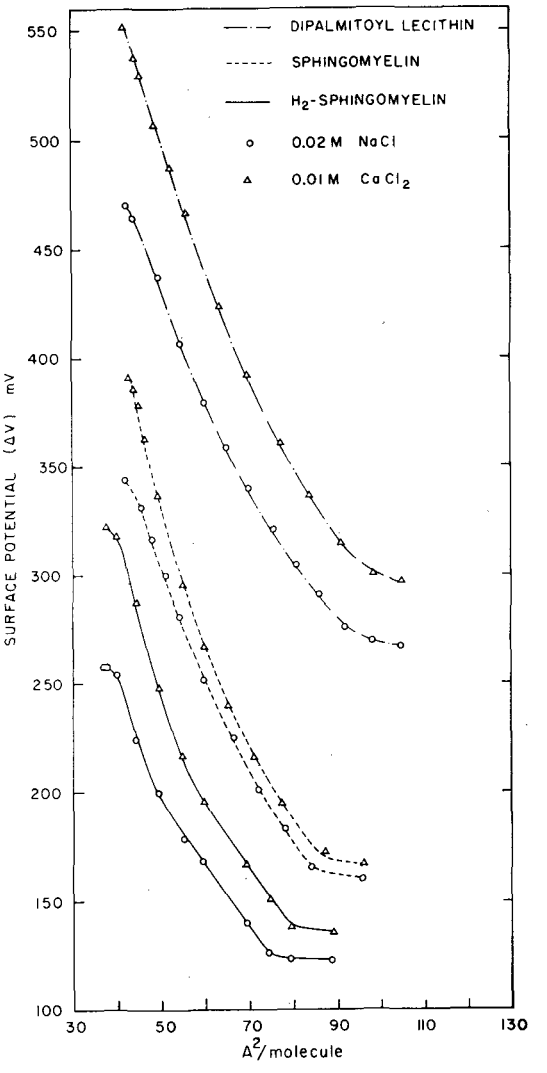

FIG. 2. Surface potential-area curves of dipalmitoyl lecithin, sphingomyelin, and hydrogenated sphingomyelin on 0.02 M NaCl (○) and 0.01 M $CaCl_2$ (△) subsolutions, pH 5.6 and 25C.

equal to that of the polar group; but if situated farther away, the double bond is not influenced by the polar group (e.g., $\triangle^6$-octadecenoic acid) (16).

In general, lipid monolayers show higher surface potentials upon hydrogenation due to an increase in the number of molecules per square centimeter of film, since a saturated hydrocarbon chain has a smaller cross sectional area compared to an unsaturated one (17), e.g., surface potentials of stearic acid vs. oleic acid (18,9) saturated vs. unsaturated

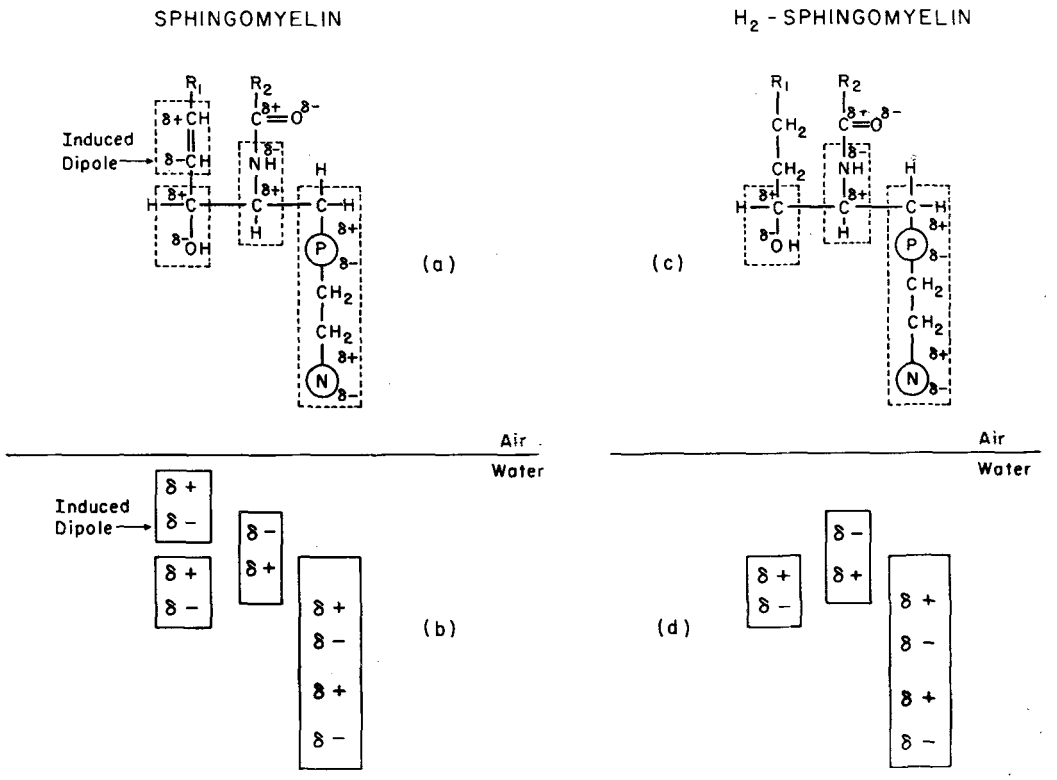

FIG. 3. The component dipoles of sphingomyelin (a) and hydrogenated sphingomyelin (c); the corresponding vertical component of individual dipole is shown in (b) and (d). R_1 and R_2 represent the hydrocarbon chains. P and N represent the phosphate and the trimethylammonium groups. $\delta+$ and $\delta-$ are the *partial* charges on the atoms.

lecithin (4), and tripalmitin vs. triolein (19). On the other hand, hydrogenated sphingomyelin shows a lower surface potential as compared to natural sphingomyelin (Fig. 2). This can be explained by a consideration of component dipoles of sphingomyelin as follows.

Nonpolar hydrocarbon chains are repelled by water and tend to be oriented approximately vertically at moderate surface pressures.[1] The polar group tends to dissolve in the subsolution and to orient itself perpendicular to the surface. On the basis of this vertical orientation of sphingomyelin molecules at the interface, the vertical components of the individual dipoles are shown in Figure 3 (a,b). It should be emphasized that Figure 3 is a simplified representation of the *vertical component* of individual dipoles, and not a scaled diagram of the molecule. However, the polarity of each component dipole is the same as that observed in the scaled three-dimensional model of sphingomyelin.

The total surface dipole of sphingomyelin can be considered as the resultant of four component dipoles as shown in Figure 3 (a,b). Consider the dipole of the phosphoryl choline group around C–1 position. Since the phosphate group and the trimethylammonium group are separated by two methylene groups, and the phosphate group is linked to the C–1 position, their dipoles can be considered individually. These dipoles are taken to be the same as those found for alkyl phosphate and alkyl trimethylammonium monolayers respectively.

[1] The inclination of the hydrocarbon chains from the vertical can be estimated as follows. Consider the hydrocarbon chains of the molecule oscillating in a cone with apex at the interface and base at the terminal of the chain. By taking the area per molecule, A, at a moderate surface pressure as the area of the base, calculate the radius r of the base. The maximum angle of inclination, Θ, of the hydrocarbon chain from vertical can be expressed by the relation $\sin \Theta = r/L$, where L is the length of the hydrocarbon chain. At a surface pressure of 20 dynes/cm, A=55 A^2/molecule; from this r is calculated to be 4.2 A. Assuming an average chainlength of 20 carbon, L = 26 A and $\sin \Theta = 0.16$, which gives the angle of inclination to vertical $\Theta = 9°$. This simplified calculation indicates that the hydrocarbon chains are almost vertical at moderate surface pressures.

Surface potential measurements of ionized or non-ionized monolayers of long chain phosphate esters (20) indicate that the phosphate group is equivalent to a resultant dipole with the upper pole positive and the lower pole negative.[2] It has been shown (21–23) that charged or uncharged alkyl amines as well as substituted amines, similarly give rise to a resultant dipole with the upper pole positive, and the lower pole negative. Since they have the same polarity, the net magnitude of this dipole is the sum of the phosphate and the trimethylammonium dipoles.

The dipole around the carbon-2 position can be estimated as follows. Since the hydrocarbon chain is approximately perpendicular to the surface, the ketonic group $C(\delta+)=O(\delta-)$ of the amide linkage is approximately horizontal (24,4) and therefore has a negligible vertical component. The linkage $C(\delta+)-N(\delta-)$ is a strong dipole with the lower pole positive; this is indicated by the C-N dipole of monolayers of alkyl amines (21–23).

The third dipole, formed by carbon-3 and oxygen of the hydroxyl group, has an upper positive pole and a lower negative pole. The double bond adjacent to this dipole acts as an induced dipole with a negative lower pole due to the induction by the third dipole.

The total dipole of the sphingomyelin molecule is the vectorial sum of these four component dipoles. Since the polarity of the second dipole $C(\delta+)-N(\delta-)$ is opposite to the rest of the dipoles, a subtraction of the second from the other three will give the total dipole of a sphingomyelin molecule. This is illustrated in Figure 3(b).

As shown in Figure 3 (c,d), hydrogenated sphingomyelin does not have an induced dipole due to the absence of the double bond. The subtraction of the second dipole from the other two dipoles will give the total dipole of the molecule. Since sphingomyelin (Figure 3 a,b) has three component dipoles with the same polarity, whereas the hydrogenated sphingomyelin has only two dipoles, the total dipole of hydrogenated sphingomyelin is smaller than that of the natural sphingomyelin. Because surface potential is directly related to the dipole of the molecule, the hydrogenated sphingomyelin should have a smaller surface potential than natural sphingomyelin. This was shown by the surface potential of hydrogenated sphingomyelin (Fig. 2).

It is to be stressed that the difference between the surface potentials of natural and hydrogenated sphingomyelin (about 90 mv at 42 A^2 per molecule on subsolutions of NaCl) cannot be due to changes in orientation of the polar group, since the molecules are closely packed and almost vertically oriented at this surface pressure. Moreover this decrease in surface potential upon hydrogenation cannot be explained on the basis of unsaturation of hydrocarbon chains, because hydrogenation would *increase* the surface potential by reducing the area of the molecules (and thereby increasing the surface concentration of molecules); in contrast, hydrogenation results in a *decrease* of surface potential of sphingomyelin (Fig. 2). Thus, the consideration on the basis of an induced dipole in 4–5 position of sphingomyelin is the only satisfactory explanation of the results presented here.

Interaction of Calcium

The *interaction* (or binding) of metal ions with negatively charged groups in the monolayer changes the electrical charge at the interface, resulting in a change of surface potential (4). For example, the presence of Ca^{++} in the subsolution does not cause a change in the surface potential of cholesterol monolayers (11) since Ca^{++} does not interact with hydroxyl groups of the monolayers. In contrast, the interaction of Ca^{++} with monolayers of alkyl phosphate (4) or fatty acids (pH>7) (9,10) results in a change of surface potential of as much as 100 mv. Direct evidence showing that metal ions do bind to fatty acid monolayers has been given by Langmuir and Schaefer (25) as well as by Schulman and Dogan (26), who removed the monolayers from the interface and subjected them to chemical analysis. In the case of phospholipids the interaction of Ca^{++} can be indicated as $2PO^-_4 + Ca^{++} \rightleftarrows PO^-_4 - Ca^{++} - PO^-_4$. At high salt concentration the amount of bound Ca^{++} would presumably approach a stoichiometric ratio (namely one Ca^{++} for two lecithin). As such, the change in surface potential does not indicate a stoichiometric ratio but only indicates an interaction between metal ions and the monolayer. The extent of this interaction is shown by the magnitude of the change in the surface potential.

We reported previously (4) that the binding of Ca^{++} to monolayers of dicetyl phosphate and lecithins results in an increase in the surface

[2] The surface potential, ΔV, is expressed as $\Delta V = V_f$ minus V_o, where V_f is the interfacial potential in the presence of film and V_o is that without the film. If ΔV is positive, then the monolayer is equivalent to a lattice of dipoles with the upper pole positive and the lower pole negative; if ΔV is negative, then the dipole has the upper pole negative and the lower pole positive.

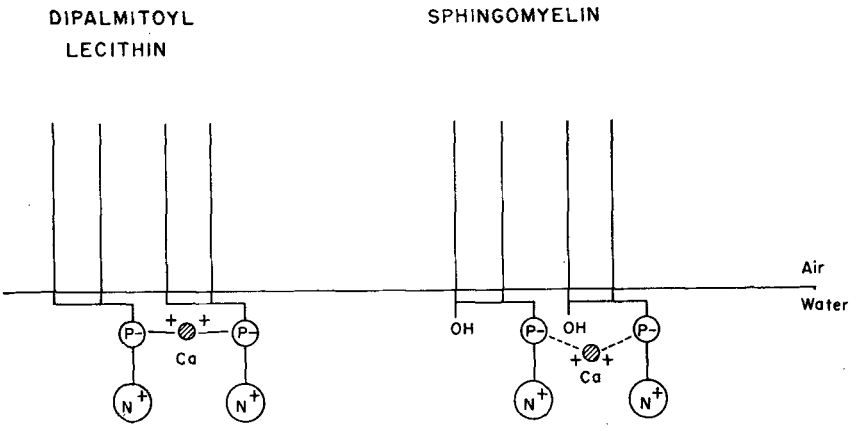

FIG. 4. Schematic representation of the interaction of calcium ion with dipalmitoyl lecithin and sphingomyelin monolayers. The dotted line represents weak and the continuous line, strong Ca^{++} interaction.

potential of the monolayers; and that the interaction with Ca^{++} is significantly influenced by a change in the intermolecular distance in the monolayers. The increase in surface potential due to the presence of Ca^{++} in the subsolution is greatest for dipalmitoyl lecithin and smallest for unhydrogenated sphingomyelin (Fig. 2). Since the limiting areas of sphingomyelin and dipalmitoyl lecithin are the same the intermolecular spacing between the phosphate groups is also the same. Figure 4 schematically shows the interaction of Ca^{++} with lecithin and sphingomyelin monolayers. The binding of Ca^{++} is less for sphingomyelin monolayers, as shown by the increase in surface potentials (Fig. 2), due to the presence of a hydroxyl group in the 3-carbon position which first, causes a steric hindrance for the two phosphate groups to share a calcium ion and second, reduces the interaction with Ca^{++} by ion-dipole association between hydroxyl and ionic phosphate groups.[3] Upon hydrogenation, sphingomyelin shows a smaller limiting area which corresponds to a smaller intermolecular spacing in the monolayer. Since the interaction between Ca^{++} and the phosphate groups of sphingomyelin is coulombic, it varies directly with the product of charges and inversely with the square of the distance between them. The smaller intermolecular spacing (due to smaller limiting area) in hydrogenated sphingomyelin monolayers increases the interaction of Ca^{++} with the phosphate groups and results in an additional increase of surface potential on subsolutions of $CaCl_2$ as compared to that of unhydrogenated sphingomyelin monolayers (Fig. 2). We have previously shown (4) that the binding of calcium to phosphate groups of lecithin is strikingly reduced by the unsaturation of fatty acyl chains. This paper reports that the presence of a hydroxyl group vicinal to the phosphate group also reduces the binding of calcium to sphingomyelin monolayers.

[3] Recent work (unpublished) done in this laboratory by Bhupendra Shah on the microelectrophoresis of aqueous dispersion of sphingomyelin showed that the particle surface possesses a net positive charge, whereas lecithin particles are known to show a zero net charge. In lecithin, the phosphate and trimethylammonium groups act as counterions for each other which results in a zero net charge. On the other hand, in sphingomyelin, the ion-dipole association between the hydroxyl and ionic phosphate groups prevents the counterbalancing effect of the phosphate on the trimethylammonium group. This results in a net positive charge on the surface of sphingomyelin particles. This positive surface charge of sphingomyelin is also supported by additional evidence from surface potentials of sphingomyelin monolayers on subsolutions of different electrolyte concentrations.

ACKNOWLEDGMENTS

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Mechanism of Lipoxidase Reaction. I. Specificity of Hydroperoxidation of Linoleic Acid[1]

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ABSTRACT

Linoleate hydroperoxides from autoxidation of methyl linoleate and from lipoxidase oxidation of linoleic acid are compared. Data indicate an equal amount of methyl 9- and 13-hydroperoxyoctadecadienoate produced by autoxidation of methyl linoleate, and the exclusive formation of 13-hydroperoxyoctadeca-9,11-dienoic acid from the incubation of lipoxidase with linoleic acid. As a result of these findings, a specific mechanism for the reaction of lipoxidase with linoleic acid is postulated.

INTRODUCTION

IN 1945–46 PRODUCTS from the autoxidation of linoleic acid were reported to be the same as those from the oxidation of linoleic acid by lipoxidase (1–3). Enzymatic oxidation of linoleic acid was thought to follow the same general course as ordinary metal-catalyzed autoxidation. The quantitative determination and identification of the 9- and 3-hydroperoxides were based on comparison of melting points (mp). Since it was subsequently shown (4) that different isomers of hydroxystearates can form eutectic systems, this identification based on mixed melting points was questionable.

In the latest review on lipoxidase (5), the mechanism of reaction is thought to involve the formation of a free radical on C-11, which explains the possibility for random formation of the 9- and 13-hydroperoxide isomers. This mechanism is strongly supported by other evidence like the presence of free radical intermediates during lipoxidase-catalyzed oxidation of linoleic acid (6–8). Lipoxidase is highly specific for oxidation of *cis, cis*-1,4-pentadiene-containing fatty acids (9) and formation of optically active conjugated *cis,trans* diene hydroperoxides. Presumably the isomerized double bond is the one converted to the *trans* configuration.

During the preparation of this manuscript, other workers pointed out (10) that lipoxidase-catalyzed hydroperoxidation of unsaturated fatty acids is relatively specific with regard to carbon atom attacked. They demonstrated that during lipoxidase-catalyzed oxidation of linoleic acid, 70% of the products formed is 13-hydroperoxyoctadecadienoic acid and only 30%, 9-hydroperoxyoctadecadienoic acid. Our work shows an even higher specificity of lipoxidase; i.e., the exclusive formation of 13-conjugated hydroperoxide by lipoxidase. Consequently, a specific mechanism for the reaction of lipoxidase with linoleic acid is justifiable and is postulated.

EXPERIMENTAL

Preparation of Hydroxystearates

From Incubation Product of Lipoxidase. The incubation of lipoxidase with linoleic acid was done in a Parr 3910 hydrogenation apparatus connected to an oxygen tank. Linoleic acid (180 mg, The Hormel Institute, 99.8% pure) was dissolved in 10.0 ml 95% ethanol and added to 80.0 ml 0.05 M borate buffer, pH 9.0. Two drops of Antifoam B (Dow Corning Corporation) was added and eliminated foaming completely. The enzyme (10.0 mg, crystalline lipoxidase[4] Nutritional Biochemical Corporation) was supended over the reaction mixture in a small glass basket while the flask was evacuated and flushed several times with oxygen. Pressure was then adjusted to 1 atmosphere of 100% oxygen at 24C. Starting the shaker released the enzyme basket which dropped into the solution. After 60 min of incubation with continuous shaking, the reaction vessel was removed from the apparatus. A more complete description and analysis of

[1] Presented at the AOCS Meeting, Philadelphia, October 1966.

[2] This work was conducted under a Postdoctoral Resident Research Associateship established at the Northern Laboratory by ARS, USDA, in association with the National Academy of Sciences-National Research Council.

[3] No. Utiliz. Res. Dev. Div., ARS, USDA.

[4] The crystalline lipoxidase preparation employed in this study was completely specific for the 13 position of linoleic acid. However, other crystalline enzyme preparations from the same supplier showed no apparent specificity oxidizing both the 9 and 13 carbons. These observations suggest that even crystalline lipoxidase may be a mixture of two or more enzymes.

this incubating procedure will be included in a future publication.

Hydroperoxide was reduced to alcohol by the addition of 100 mg $NaBH_4$ to the reaction mixture at room temperature with magnetic stirring (11). After 60 min, excess $NaBH_4$ was decomposed by acidifying the reaction mixture to pH 3.0 with concentrated HCl. During the reduction a flow of N_2 was maintained over the reaction mixture.

The product was extracted three times with 50 ml diethyl ether, residual water in extract was removed with Na_2SO_4 and extract evaporated to about 25 ml under reduced pressure in a rotatory evaporatory at room temperature. After methylation in diethyl ether with diazomethane, the solvent was evaporated under a stream of N_2 and the residue removed under reduced pressure. Hydrogenation of the products took place in a microhydrogenator with absolute ethanol as solvent and 5% palladium on carbon as catalyst.

From Autoxidized Methyl Linoleate. Methyl linoleate (4.3 g and 99.6% pure) prepared by counter double current distribution (12) was placed in a 20-cm long 0.5 cm inside diameter test tube kept at 40C with oxygen bubbling through at a low rate. After the peroxide value (PV) reached a level of 2400, 300 mg of the autoxidized methyl linoleate was dissolved in 10 ml 95% ethanol, 80 ml 0.05 M borate buffer, pH 9.0, was added and the hydroperoxide was converted to an alcohol by reducing it with $NaBH_4$. The mixture was acidified, extracted with diethyl ether and hydrogenated catalytically.

Separation of Products

Column chromatography on high-grade silica gel (Adsorbosil, Applied Science Laboratories) was used. The quantitative separation between methylstearate, methyl keto stearates, methyl 9-hydroxystearate and methyl 13-hydroxystearate has been described previously (13). The identity and purity of the last two compounds were verified by comparing the analysis with that of known pure isomers by gas-liquid chromatography (GLC), thin-layer chromatography (TLC), infrared (IR), mass spectroscopy and melting points. GLC was performed on a Pye argon gas chromatograph equipped with a 4 ft × ¼ in. glass column packed with 14% EGSS-X on Gas-Chrom P, 100/200 mesh (Applied Science Laboratories) and with a radium D ionization detector. Column temperature was 170C and the argon gas flow 100 ml/min. The area under each curve was determined from

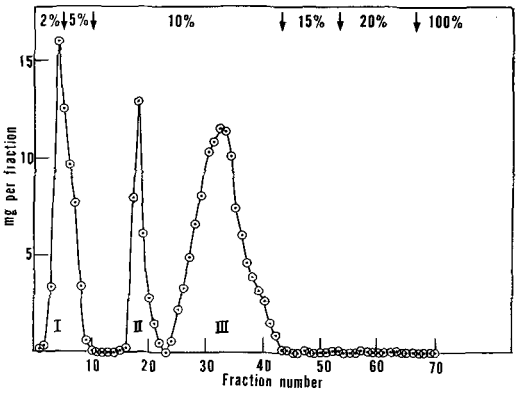


FIG. 1. Separation of lipoxidase hydroperoxidation products of linoleic acid. Methyl stearate (I), methyl keto stearate (II) and methyl 13-hydroxystearate (III) on Adsorbosil, 202 mg applied and collected in 70 fractions of 2 ml eluate each (98.2% recovery). Percentage indicated refers to concentration of diethyl ether in *n*-hexane.

an electronically integrated curve. Glass plates (20 × 20 cm) spread with a 0.2-mm layer of Silica Gel G (Brinkmann Instruments, Inc.) and activated for 30 min at 110C were used for TLC analysis. Hexane: diethyl ether (7:3) was the developing solvent. Infrared analysis was performed in a Model 621 Perkin-Elmer spectrophotometer. The mass spectra were measured on a Nuclide 12–90 G mass spectrometer equipped with an all-glass inlet; inlet

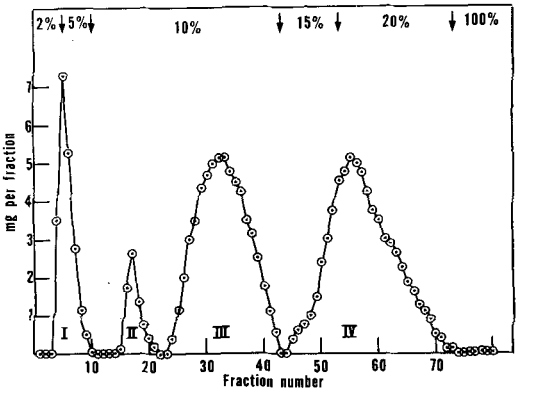


FIG. 2. Separation of autoxidation products of methyl linoleate. Methyl stearate (I), methyl keto stearate (II), methyl 13-hydroxystearate (III) and methyl 9-hydroxystearate (IV) on Adsorbosil, 155 mg applied and collected in 80 fractions of 2 ml eluate each (98.7% recovery). Percentage indicated refers to concentration of diethyl ether in *n*-hexane.

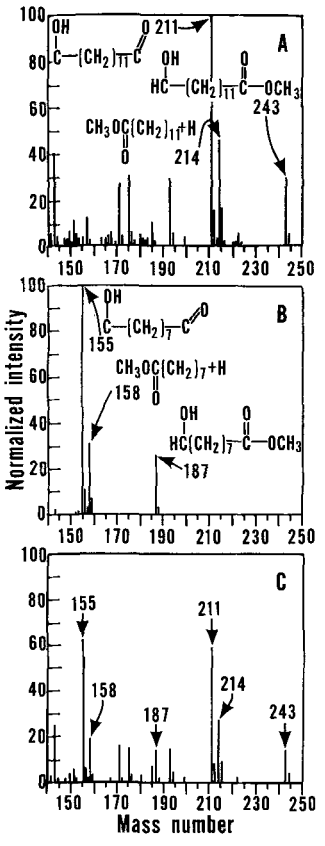


FIG. 3. Mass spectra fragmentation patterns. A is the constituent of peak III, methyl 13-hydroxystearate; B is the constituent of peak IV, methyl 9-hydroxystearate; and C is a mixture of both.

temperature, 170C; source temperature, 225C; and 70 v electron energy.

RESULTS AND DISCUSSION

Figures 1 and 2 show the separation of the different reduced products of lipoxidase oxidation and autoxidation of linoleic acid on the silica gel column. Only peaks III and IV had a characteristic hydroxy band at 3646 cm^{-1} as determined by IR. Both had the same retention time on GLC and it corresponded to the retention time of methyl 9-hydroxystearate (13). The respective mp's of products corresponding to peaks III and IV were 50.0–50.5C and 47.5–48.0C.

The mass spectra of the isomeric methyl hydroxystearates show characteristic fragmentation patterns that result from cleavage of the carbon-carbon bonds on each side of the carbon atom to which the hydroxy group is attached. This fragmentation pattern for known pure methyl 8-, 9-, 10, 12, and 13-monohydroxystearates is consistent with the interpretation of the methyl 8- and 10-monohydroxystearates given by Ryhage and Stenhagen (14). The various mass peaks are interpreted in Figure 3. The fragmentation pattern of the constituent of peak III (Fig. 3A) has a mass base peak 211, whereas the constituent of peak IV (Fig. 3B) gives a mass base peak 155. The mass spectrum of methyl 9-hydroxystearate from the hydrogenation of methyl dimorphecolate was identical with that of peak IV. Therefore, the constituents of peaks III and IV are the 13- and 9-hydroxystearates, respectively. The mass spectra of the methyl hydroxystearates from the reduction of the product of autoxidation of methyl linoleate are identical with the mass spectra of the mixture of both isomers. The fragmentation patterns of the isomers are quite different, but each is consistent and can be easily distinguished. Contamination of one of the isomers with the other can be clearly detected from the mass spectra of the mixture.

The quantitative analysis of the autoxidation products of methyl linoleate and lipoxidation products of linoleic acid is presented in Table I. As previously shown (15), hydroperoxides produced by the autoxidation of methyl linoleate form about equal amounts of the 9- and 13-isomers. Completely unexpected were the findings from the analysis of the lipoxidase oxidation products of linoleic acid. In several different incubations of lipoxidase with linoleic acid only the methyl 13-hydroxystearate was isolated, and there was no evidence of methyl 9-hydroxystearate. Possibly the 9-isomer might have been lost during the separation procedure, but autoxidation products of methyl linoleate carried through the same procedure yielded the expected 9- and 13-hydroxystearates.

Unless two different specific oxidative enzymes are present in soybean lipoxidase (8,16), one specific for the formation of the 9-hydroperoxide isomer and the other specific for the 13-isomer, the formation of both isomers in unequal amounts is difficult to explain (10). Explanations for the differences between our study and Hamberg and Samuelsson's might cite various factors, such as the differences in the enzymes used, different incubation and analytical procedures, and the possibility of interference by autoxidation that would produce a significant amount of the 9-isomer and obscure specificity.

TABLE I

Quantitative Analysis of Autoxidation Products of Methyl Linoleate and Lipoxidation of Linoleic Acid

Compound	Lipoxidase oxidation of linoleic acid		Autoxidation of methyl linoleate	
	Separation on column[a]	GLC determination[b]	Separation on column[a]	GLC determination[b]
Methyl stearate	26.7	24.1	13.4	11.8
Methyl keto stearate	16.3	19.1	4.8	5.6
Methyl 9-hydroxystearate			39.5	
Methyl 13-hydroxystearate	57.0	56.8[c]	42.2	82.4[c]
	100.0	100.0	99.9	99.8

[a] Percent weight of recovered product.

[b] Relative percent area.

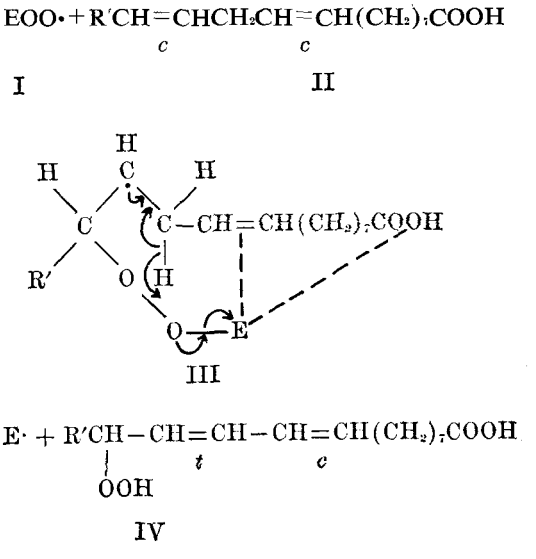
[c] The 9- and 13-hydroxystearate isomers gave one common peak on GLC.

Lipoxidase was previously demonstrated to be specific for the substrate, i.e., it attacks only a *cis,cis*-1,4-pentadiene system (9). The formation of an optically active isomer from lipoxidase hydroperoxidation (17) versus the lack of any optical activity in autoxidation products, also points out the high specificity of the enzymatic reaction.

Based on these new findings it might be well to reconsider the sequence of the reactions involved, as given in Scheme 1. From a purely mechanistic point of view one has two alternatives in explaining the synthesis of only one isomer. If one accepts the conventional mechanism proposed for the reaction of lipoxidase (5), it is difficult to account for the blocking of the C-9 position following the abstraction of the methylenic hydrogen and formation of a free radical on C-11.

Although a free radical intermediate was shown to be present in the overall reaction, it was not established to be present only in a specific step in the sequence of reactions involved nor was it assigned to a specific carbon atom. Since several oxidative enzymes, including lipoxidase, were shown to initiate free radical intermediates (8) we feel that a new mechanism is worth consideration. As shown in the scheme, we propose that a free radical adds to the substrate rather than abstracts hydrogen from it. Activation of the enzyme by oxygen forms a free radical (I) which reacts with linoleic acid (II) by addition at C-13; i.e., the unsaturated carbon atom farthest from the carboxyl group. It appears probable that I is held in the proper position to facilitate this reaction by attachment to the substrate via the double bond at C-9 and/or the carboxyl group. A series of one-electron shifts, indicated by arrows, then takes place in intermediate III. These shifts result in establishment of a new *trans* double bond at C_{10}, transfer of a hydrogen atom to oxygen to form the hydroperoxide group, and liberation of the enzyme, perhaps as the free radical E° which could react with O_2 to regenerate I. The product is, therefore, exclusively a 13-hydroperoxy-9,11-octadecadienoic acid (IV).

Investigation into the origin of the oxygen molecule incorporated into the hydroperoxide



SCHEME 1. Postulated reaction sequence of lipoxidase, oxygen and linoleic acid.

by the use of $^{18}O_2$ is completed and will be reported shortly.

ACKNOWLEDGMENTS

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Mechanism of Lipoxidase Reaction. II. Origin of the Oxygen Incorporated into Linoleate Hydroperoxide[1]

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ABSTRACT

Two different series of experiments were performed to establish the origin of the oxygen molecule incorporated into hydroperoxide during the incubation of lipoxidase with linoleic acid. These showed, as previously assumed but never demonstrated, that the oxygen introduced into the hydroperoxide molecules comes from the gaseous phase and not from the aqueous phase. Furthermore, soybean lipoxidase does not catalyze the exchange between gaseous oxygen and water oxygen. Possibly, lipoxidase may be involved in the biosynthesis of hydroxy *trans,cis* conjugated octadecadienoates present in various seeds.

INTRODUCTION

As stated in a preliminary communication (1), it has always been assumed, but never demonstrated, that the oxygen molecule, incorporated into the hydroperoxide as a result of lipoxidase-catalyzed oxidation of linoleic acid, comes from the gas phase.

Publications before 1963 on the nature, mechanism of reaction and specificity of soybean lipoxidase reactions have been summarized by Tappel (2). In 1965, Hamberg and Samuelsson (3) suggested that lipoxidase attacks the ω-6 carbon atom specifically. They showed that lipoxidase-catalyzed oxidation of linoleic acid produced 13-hydroperoxyoctadeca-9,11-dienoic acid and 9-hydroperoxyoctadeca-10,12-dienoic acid in a ratio of 7:3. Following our development of an analytical procedure that permits the quantitative separation of methyl 13-hydroxystearate from methyl 9-hydroxystearate (4), an even higher specificity for soybean lipoxidase was discovered; i.e., the exclusive formation of 13-hydroperoxyoctadeca-9,11-dienoic acid (5).

The assumed source of the oxygen molecule in lipoxidase oxidation became suspect because of observations on hydrogenation of sorbic acid (6). During the homogeneous catalytic hydrogenation of this acid with pentacyanocobaltate, the hydrogen incorporated into the hydrogenated molecule originated in the H_2O molecule and not in the H_2 gas phase as predicted.

Two kinds of experiments were performed which demonstrated that lipoxidase does not catalyze oxygen exchange between the water and gas and that the oxygen incorporated into the hydroperoxide molecule by lipoxidase does indeed come from the gas phase.

Based on the data available from this series of investigations on the mechanism of reaction of lipoxidase, which correlate with recent advances in seed lipids chemistry, a biosynthetic role for lipoxidase is quite possible and is postulated.

EXPERIMENTAL

Two types of experiments were designed to determine where the oxygen incorporated into hydroperoxide originates: the first, incubation of lipoxidase with linoleic acid in a $H_2{}^{18}O$ buffer with $^{16}O_2$ in the gas phase; and the second, the same incubation but in normal $H_2{}^{16}O$ buffer with isotopic $^{18}O_2$ in the gas phase.

Incubation of Lipoxidase with Linoleic Acid

In $H_2{}^{18}O$ Buffer. Incubation was essentially as described previously (5), including treatment of the incubated products, reduction of the hydroperoxide with $NaBH_4$, methylation with diazomethane, reduction of the double bonds with 5% Pd on C, fractionation by column chromatography and analysis by mass spectrometry of the methyl hydroxystearate. The borate buffer was prepared from water enriched with 5.41 atom percent ^{18}O (Yeda Research and Development Co., Rehovoth, Israel).

In $^{18}O_2$ Gas Phase. A special flask (125 ml, round bottom) was designed (Fig. 1) that permitted us to introduce, through neck A, 80.0 ml of 0.05 M, borate buffer (pH 9), 180 mg linoleic acid (99.8% pure, Hormel Institute) dissolved in 10.0 ml 95% ethanol, 15 mg

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[2] This work was conducted under a Postdoctoral Resident Research Associateship established at the Northern Laboratory by ARS, USDA, in association with the National Academy of Sciences-National Research Council.

[3] No. Utiliz. Res. Dev. Div., ARS, USDA.

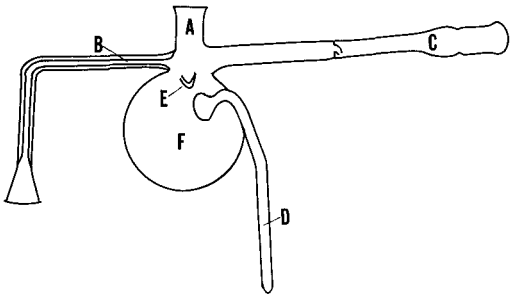

FIG. 1. Flask designed for incubation of lipoxidase with linoleic acid under $^{18}O_2$ gas: *A*, neck, 20 mm diameter; *B*, capillary tubing with joint leading to a Toepler pump; *C*, arm equipped with break seal for recovery of gas; *D*, a "cold finger" for O_2 condensation; *E*, glass hook from which to suspend glass basket with enzyme; *F*, round bottom flask, 125 ml.

crystalline lipoxidase (Nutritional Biochemical Corporation) suspended in a glass basket on glass hook E inside the flask and a small Teflon-coated magnet. The flask was then immersed in a −80C mixture of dry ice-methyl Cellosolve. After the contents of the flask were frozen, neck A was sealed. Neck B was then connected to a Toepler pump, which, in turn, was connected to a high-vacuum mercury-diffusion pump manifold. The whole system was then evacuated to 10^{-3} mm Hg. A Dewar flask filled with liquid N_2 was then raised around "cold finger" D to condense oxygen as it was introduced by the Toepler pump and to permit transfer of 100 ml oxygen into about a 45 ml headspace. The break seal on the $^{18}O_2$ reservoir (100 ml, 91.6 atom percent ^{18}O, Yeda, Rehovoth, Israel) was then broken with a magnet and the gas transferred into the reaction flask by 11 strokes of the Toepler pump. On the final cycle of the Toepler pump, the mercury was allowed to rise into the capillary joint and the tubing (neck B) was sealed off at 1 cm above the mercury.

Contents of the flask were thawed and brought up to room temperature by swirling carefully in a 25C water bath. After the basket of enzyme was released into the substrate, the mixture was stirred with a magnetic stirrer for 60 min. The sealed flask was again placed in the −80C mixture, frozen and connected through arm C to the high-vacuum manifold. After a 10^{-3} mm Hg pressure was reached, the seal on arm C was broken with a magnet and the gas phase over the reaction mixture was pumped through the Toepler pump and collected in another flask, also immersed in liquid N_2. This recovered gas was later analyzed by mass spectrometry. The reaction mixture was treated and carried through the same sequence of reactions as the products of the incubation of lipoxidase with linoleic acid in $H_2{}^{18}O$ buffer.

Analytical Procedures

Gas-liquid (GLC), thin-layer (TLC), and column chromatography, infrared (IR) and mass spectrometry were performed as described earlier (5). Ultraviolet (UV) spectra were taken on a Cory 14 recording spectrophotometer. The following analyses were performed before and after hydrogenation: GLC, TLC, IR and UV.

RESULTS AND DISCUSSION

The results of Experiment 1, in which lipoxidase was incubated with linoleic acid in a buffer with either enriched $H_2{}^{18}O$ or $H_2{}^{16}O$ under 1 atmosphere of $^{16}O_2$ gas, are presented in Table I. About 70–75% of the substrate in both incubations was recovered as 13-hydroperoxy-9,11-octadecadienoic acid and the rest as the original linoleic acid. No ^{18}O was de-

TABLE I

Analysis of Products from Incubation of Linoleic Acid in $H_2{}^{18}O$ and $H_2{}^{16}O$ with Lipoxidase and $^{16}O_2$ Gas

Product	Separation on column[a]	GLC determination[b]	IR a_{3640} cm^{-1}	Mass spectrometry analysis ^{16}O[c]	Mass spectrometry analysis ^{18}O[c]
In $H_2{}^{18}O$ buffer					
Methyl stearate	26.6	24.1	....	100	....
Methyl 13-ketostearate	19.8	19.1	....	100	....
Methyl 13-hydroxystearate	53.6	56.8	0.1651	100	....
In $H_2{}^{16}O$ buffer					
Methyl stearate	25.1	25.7	....	100	....
Methyl 13-ketostearate	18.2	15.6	....	100	....
Methyl 13-hydroxystearate	56.7	58.7	0.1665	100	....

[a] Weight %.

[b] Area %.

[c] Atom %.

tected in the final reduced product; i.e., methyl 13-hydroxystearate. Hence, lipoxidase cannot utilize oxygen atoms from water in forming hydroperoxide.

The analytical results of Experiment 2, in which lipoxidase was incubated with linoleic acid in $H_2{}^{16}O$ buffer and under $^{18}O_2$ gas, are given in Table II and Figure 2.

Oxygen pressures varied during these reactions because 100 ml oxygen (at STP) was introduced into a 45 ml headspace. Since about 15 ml oxygen was incorporated into the hydroperoxide molecules, there was a pressure drop from 2.2 atmospheres to 1.9 atmospheres during the reactions. In spite of this pressure change, there was no significant difference between the products produced in Experiments 1 and 2. Also, the control run with $^{16}O_2$ gas yielded the same product composition as the run with $^{18}O_2$. Analysis of the products showed that no ^{18}O was present in the methyl stearate fraction. Both methyl 13-hydroxystearate and methyl 13-ketostearate (hydrogenation byproduct, see below) contained more than 80 atom percent ^{18}O (84.0 and 81.8%, respectively). The difference between the ^{18}O enrichment in the final products and the level of ^{18}O in the gas used can be attributed mainly to the $^{16}O_2$, which remained dissolved in the buffer. In the mass spectrum (Fig. 2), all fragments containing hydroxyl oxygen (5) are seen to have shifted two mass numbers higher than the corresponding ^{16}O fragments.

As suspected, the methyl 13-ketostearate is a byproduct of the hydrogenation of methyl 13-hydroxy-9,11-octadecadienate. The following observations support this statement: No ketone fraction is present in the methylated incubation product of lipoxidase with linoleic acid; yet after catalytic hydrogenation of the incubation products, about 18% of the original linoleic acid is converted to methyl 13-ketostearate. In addition, the keto group has the same level of ^{18}O enrichment as the alcohol group in the methyl 13-hydroxystearate fraction.

Experiment 2 establishes that the oxygen molecule incorporated into the hydroperoxide comes from the gas phase.

IR spectrum of the methyl 13-hydroxystearates indicated a strong, sharp hydroxy peak at 3640 cm^{-1}. Introducing ^{18}O instead of ^{16}O into the molecule did not cause any change in wavelength of the hydroxy peak (Table I). The hydroxystearate from reduction of the hydroperoxide produced by lipoxidase oxidation of linoleic acid exhibited optical activity in contrast to the hydroxystearate from autoxidation of methyl linoleate.

TABLE II

Analysis of Products from Incubation of Linoleic Acid with Lipoxidase with $^{18}O_2$ or $^{16}O_2$ in $H_2{}^{16}O$ Buffer

Product	^{16}O[a]	^{18}O[a]	Total incubation product Composition[b]
With $^{16}O_2$ gas			
Methyl stearate	100	....	24.7
Methyl 13-ketostearate	100	....	18.9
Methyl 13-hydroxystearate	100	....	56.4
With $^{18}O_2$ gas			
Methyl stearate	100	....	24.1
Methyl 13-ketostearate	14.2	81.8	18.2
Methyl 13-hydroxystearate	14.1	84.0	57.7

[a] Atom %.
[b] Weight %.

Mass spectrometry analysis of the recovered gas after the $^{18}O_2$ incubation, indicated essentially the same composition of the original gas; i.e., 90.7 atom percent ^{18}O and 9.3 atom percent ^{16}O.

If lipoxidase had catalyzed the exchange of oxygen between the aqueous and the gaseous phases, some of the aqueous heavy oxygen would have been present in the gaseous oxygen and incorporated by the enzyme into the hydro-

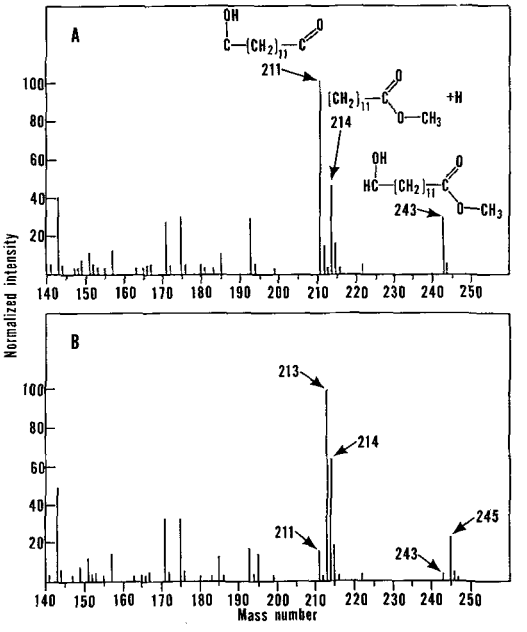

FIG. 2. Mass spectrometry fractionation pattern of methyl 13-hydroxystearate recovered from the reduced incubation products of lipoxidase with linoleic acid under $^{16}O_2$ gas (A) and under $^{18}O_2$ gas (B). The hydroxyl group containing fragments are two mass units higher in B than in A, owing to the ^{18}O incorporation.

peroxide. Since no isotopic ^{18}O was found in the methyl 13-hydroxystearate produced by Experiment 1, lipoxidase does not catalyze this exchange.

In recent publications by Ryhage, Samuelsson and Hamberg (7–9), essentially the same procedure was used to study the incorporation of oxygen in the conversion of 8,11,14-eicosatrienoic acid to prostaglandin E_1. They suggest that the introduction of a hydroxy group to C-15 of prostaglandin E_1, which involves also the isomerization of a double bond, is analogous to lipoxidase oxidation. In a review on seed lipids, Wolff (10) names the different hydroxy fatty acids in seeds containing exclusively 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid, 9-hydroxy-*trans*-10,*cis*-12-octadecadienoic acid, or a mixture of both. The positions of the hydroxyl group (2,5,10), the optical activity of those compounds, the conjugated diene system, and the *trans* configuration of the unsaturated bond nearest the hydroxyl group, all point to the possibility that lipoxidase may be involved in the biosynthesis of these hydroxy *trans,cis* conjugated octadecadienoates.

ACKNOWLEDGMENT

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Quantitative Analysis of Brain and Spinach Leaf Lipids Employing Silicic Acid Column Chromatography and Acetone for Elution of Glycolipids

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ABSTRACTS

Quantitative elution of acidic and neutral glycolipids of brain and spinach leaves from silicic acid columns with acetone was demonstrated. Cerebrosides and sulfatides of brain and sulfolipid and glycosyl diglycerides of spinach leaves were eluted quantitatively with acetone while prospholipids remained on the column. The observations provide the basis for an analytical procedure employing column and quantitative thin-layer chromatography (TLC). Sephadex column chromatography is utilized for separation of lipids from nonlipids; silicic acid column chromatography for separation into neutral lipid, glycolipid and phospholipid fractions; and quantitative TLC for analysis of lipid classes of each column fraction.

INTRODUCTION

While acetone has been associated with the purification of lipid classes since the late nineteenth century, it has been used infrequently as an elution solvent in chromatographic separations. Previously, Smith and Freeman (1) used elution with acetone for the separation of cerebrosides from milk, and Nelson and Freeman (2) used acetone for elution of oxidation products of less polar lipids prior to elution of polar lipids with chloroform/methanol mixtures. Recently Vorbeck and Marinetti (3) reported separation of glycosyl diglycerides of bacterial lipids by elution of silicic acid columns with chloroform/acetone and acetone. In the present studies the elution characteristics of acetone for brain and spinach lipids were determined. The results of the studies are presented in this report and an analytical procedure employing Sephadex column chromatography, silicic acid column chromatography with elution of glycolipids with chloroform/acetone and/or acetone, and quantitative thin-layer chromatography (TLC) is described.

MATERIALS AND METHODS

Solvents

Reagent grade chloroform, acetone, and methanol were redistilled from glass.

Extraction and Removal of Nonlipid Contaminants

Lipids were extracted with chloroform/methanol 2/1 from human brain and fresh spinach leaves (4) and the extracts freed of nonlipid contaminants by column chromatography on Sephadex (5). The lipid eluted from Sephadex with chloroform/methanol 19/1 saturated with water was used for silicic acid column chromatography.

Silicic Acid Column Chromatography

Unisil (100-200 mesh, Clarkson Chemical Co., Inc., Williamsport, Penna.) without additional treatment was transferred in chloroform to chromatography tubes of different sizes equipped with Teflon stopcocks, solvent reservoirs, and glass wool plugs for retention of adsorbent. All samples were applied in chloroform. Columns 2.5 cm in diameter were eluted at a flow rate of 3 ml/min. larger or smaller columns being eluted at equivalent rates based upon ratios of surface areas. Fractions equivalent to one column volume were collected and solvent was removed by evaporation at low temperature. Solids were dissolved in 1-3 ml of chloroform/methanol 2/1 and examined by one- or two- dimensional TLC (6,7) for identification of components.

The less polar (neutral) lipids were first eluted with 8 column volumes of chloroform. Chloroform/acetone 1/1, acetone, and acetone/water mixtures (containing 0.5, 1.0, 5.0 and 10.0% water) were used for elution of glycolipids and then methanol was used to clear columns of phospholipid.

Quantitative TLC

One-dimensional TLC with the charring-transmission densitometry procedure (7,8) was

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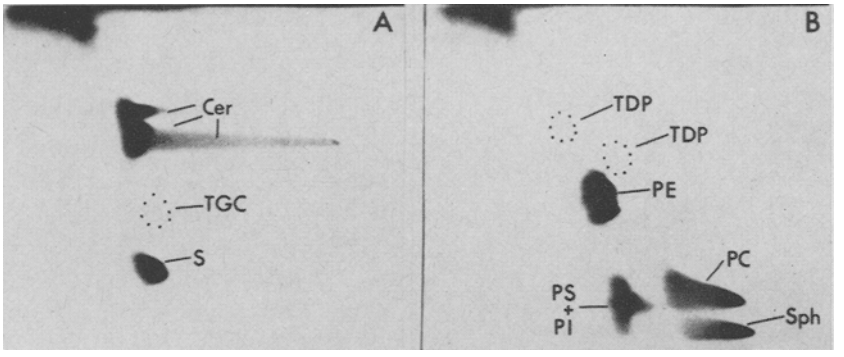

FIG. 1. Two-dimensional TLC of the mixture of glycolipids eluted with acetone (1A) and phospholipids eluted with methanol (1B) from a silicic acid column with brain lipid as sample. 200 μg of glycolipid and 400 μg of phospholipid was applied to the lower right corner of the plate and the chromatograms were developed first (vertical direction) with chloroform methanol/water 65/25/4, dried for 10 min, and developed (horizontal direction) with 1-butanol /glacial acetic acid/water 60/20/20. Spots were visualized with the sulfuric acid-potassium dichromate char spray and heat (6). At the high sample load used for 1A cerebroside (Cer) streaks in the second solvent system.

Abbreviations: Cer, cerebroside; S, sulfatide; TCG, trace glycolipid components (probably ceramide dihexoside and diglycosyldiglyceride); TDP, trace decomposition products from phospholipids; PE, phosphatidyl ethanolamine; PS + PI, phosphatidyl serine plus phosphatidyl inositol; PC, phosphatidyl choline; Sph, sphingomyelin.

used for neutral and glycolipids. Two-dimensional TLC followed by aspiration of spots and phosphorus analysis was used for phospholipids (10).

RESULTS AND DISCUSSION

General Glycolipid Elution Characteristics

Acetone eluted both cerebrosides and sulfatides of brain and glycosyl diglycerides and sulfolipid of spinach leaves. Chloroform/acetone 1/1 completely separated spinach leaf monoglycosyl diglyceride from diglycosyl diglyceride and the latter was elutable with acetone in keeping with the findings of Vorbeck and Marinetti (3) for bacterial glycosyl diglycerides. Chloroform/acetone 1/1 eluted cerebrosides, but sulfatides began to appear in the effluent before the last trace of cerebroside was eluted. Elution with chloroform/acetone 1/1 is thus advantageous with spinach lipids, but is not as useful for quantitatitive analysis of brain lipids. Since cerebroside is almost completely eluted with chloroform/acetone before sulfatide appears in the effluent, elution of cerebrosides with chloroform/acetone 1/1 is useful as a preparative procedure. Small amounts of water (0.5-1.0%) in acetone did not appreciably increase the elution rate of glycolipids, while water at 2-10% levels decreased the elution volume for glycolipids but brought about elution of phospholipids. Acetone/water mixtures thus do not possess advantages over acetone alone for glycolipid elution.

Methanol eluted phospholipids and any material on the column not removed with either chloroform or acetone. In all chromatographic runs recoveries were 100 $\pm$ 1% of the sample applied to the column.

Columns 5, 10, and 20 cm in height were compared. Columns 5 cm in height were satisfactory for both brain and spinach lipids. Usually 100 mg of lipid was applied to columns 2.5 cm in diameter, although smaller loads were equally satisfactory.

Final Silicic Acid Column Procedure and Results with Brain Lipids

Columns 2.5 (I.D.) by 5 cm were used. The sample (100 mg) was applied in chloroform (5-10 ml). Such columns were found to have a column volume of about 17.5 ml. Cholesterol and free fatty acids were eluted with 8 column volumes of chloroform. Acetone (40 column volumes) was then used for elution of cerebrosides, sulfatides, and a trace component, also apparently glycolipid. Phospholipids were then eluted with 10 column volumes of methanol.

The three glycolipid components of the acetone fraction of brain observed by TLC are shown in Fig. 1A. The acetone fraction was devoid of phosphorus. The methanol eluate from brain lipid contained only minute traces of cerebroside and sulfatide as judged by visual examination after TLC, as shown in Fig. 1B.

The amounts of cholesterol and glycolipid

eluted with chloroform and acetone, respectively, using human brain samples, were in good agreement with values obtained previously for normal adult human brain (9). With two different specimens of adult male brain the values as weight percentages of the crude chloroform/methanol extract prior to removal of gangliosides and nonlipid contaminants were: cholesterol 22.3 and 22.1 and cerebrosides plus sulfatide 21.7 and 22.0 by silicic acid column chromatography and the DEAE-TLC procedures, respectively. The ratio of cerebroside to sulfatide was determined by the charring-transmission densitometry procedure (6) and found to be 2.60 for glycolipid eluted from silicic acid with acetone, in good agreement with the ratio 2.56 (from values of 15.8 and 6.2% for cerebroside and sulfatide, respectively) previously reported for whole brain of a normal adult (9).

Trace Components of Brain Eluted with Acetone

The nature of the trace component(s) of brain eluted with acetone (Fig. 1A) was investigated in a preliminary manner. A small amount of the trace component(s) was isolated from TLC plates. Two spots migrating very close together were obtained by TLC in several solvents. Both spots were positive to the α-naphthol spray for glycolipids (5) and their migration in several solvents corresponded to digalactosyl diglyceride and a synthetic sample of ceramide lactoside. When chromatographed with chloroform/methanol/28% aqueous ammonia 65/35/5 on plates spread with 0.025 M sodium borate instead of water, the migration of both spots was greatly reduced as observed for glycolipids. The infrared spectrum showed both ester and amide bands suggesting the presence of a mixture of diglycosyl diglyceride and ceramide dihexoside. This possibility was further strengthened by the observation that part of the mixture was labile to mild alkaline hydrolysis, and sphingosine and fatty acid (identified by TLC) were released by acid hydrolysis.

Final Silicic Acid Column Procedure and Results for Spinach Leaf Lipids

Lipid (100 mg) was applied in chloroform (5-10 ml) to a 2.5 (I.D.) by 5 cm column. Eluting solvents were: 1) chloroform (8 column volumes) for neutral lipids; 2) chloroform/acetone 1/1 (6 column volumes) for monoglycosyl diglycerides along with small amounts of somewhat less polar lipids; 3) acetone (35 column volumes) for diglycosyl diglyceride, sulfolipid and small amounts of uncharacterized lipids; and 4) methanol (10 column volumes) for remaining lipid. No attempt was made to determine the precise amounts of the several components in each fraction, although the bulk fraction weights were reproducible to within ± 2% on different runs. The methanol eluate was entirely devoid of glycosyl diglycerides.

Combined Column—TLC Procedure for Analysis of Lipid Classes

The analytical procedure based upon the above observations employs three chromatographic techniques: Sephadex column chromatography (5) for separation of lipids from nonlipids and gangliosides; silicic acid column chromatography for separation of the lipid fraction into neutral, glycolipid, and phospholipid fractions; and TLC for separation and determination of the individual lipid classes in the fractions obtained from silicic acid columns.

The Sephadex column procedure has been modified for routine use. The washing procedure used earlier (5) has been omitted and instead each newly packed column is washed with each of the four different eluting solvents before application of the sample. Columns 2.5 (I.D.) by 10 cm requiring only one-third as much solvent for elution of fractions give essentially the same performance as the 30 cm high columns used previously.

Quantitative one-dimensional TLC by the charring transmission densitometry procedure (8,9) is used for analysis of the individual lipid classes eluted with chloroform and acetone (cerebroside plus sulfatide or glycosyl diglycerides plus sulfolipid fraction). Components of the methanol eluate (phospholipids) are determined by two-dimensional TLC and phosphorus analysis of spots (10).

Other Components of Sephadex Column Fractions of Brain and Spinach

The separation of gangliosides from other brain lipids by Sephadex column chromatography was established previously, but data on plant lipids have not been reported. With spinach leaf lipids as sample, components migrating by TLC and charring like lipids were detected in Sephadex column fractions 2 and 3. The nature of these minor components of spinach leaf extracts is not known and was not investigated.

ACKNOWLEDGMENTS

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Chemical Composition of Subcellular Particles from Cultured Cells of Human Tissue [1]

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ABSTRACT

Chemical composition of subcellular components of HeLa, KB, human heart and liver tissue-culture cell lines have been studied.

The concentration of RNA, protein and phospholipid (μg/μg of DNA) of total subcellular particles was similar for all four cell lines studied. The greatest RNA concentration and lowest protein concentration is found in the microsomes as compared to the other subcellular fractions of HeLa and KB cells.

The lipid P/Protein N ratio of mitochondria was greater than the other subcellular fractions from tissue-culture cell lines studied. Phosphatidyl choline and phosphatidyl ethanolamine are the major phospholipids with the former more predominant in all of the subcellular fractions of tissue-culture cells studied. Phosphatidyl inositol, phosphatidyl serine, sphingomyelin and polyglycerol phosphatide were shown to be present. Phosphatidyl choline composition (per cent of total lipid-P) is greatest in the microsomes when compared with the other subcellular fractions obtained from all of the cell lines studied except the nuclear fraction of human liver cells. Correspondingly, the mitochondrial fraction for all of the tissue culture cell lines contains the greatest composition of phosphatidyl ethanolamine except for the human liver and heart cells. The mitochondrial fraction contains the lowest amount of phosphatidyl inositol. Polyglycerol phosphatide is mainly present in the mitochondrial fraction of the tissue-culture cells.

INTRODUCTION

TISSUE-CULTURE CELLS have been used for many purposes such as virus research, screening potential chemotherapeutic drugs, and morphological and nutritional studies. However, few experiments have been reported on chemical analyses of the subcellular fractions of tissue-culture cells. The use of chromatography, differential centrifugation and microchemical methods enables one to investigate the chemical composition of subcellular fractions of the cell. This report presents the results of experiments concerning the chemical composition of subcellular components, nuclei, mitochondria and microsomes of various tissue-culture cells.

MATERIALS AND METHODS

Cell Growth

Four kinds of heteroploid serial cultures were originally obtained from Microbiological Associates, Inc., Bethesda, Md., HeLa (human carcinoma of cervix) and KB (human carcinoma of nasopharynx) are derived from malignant tissues; human adult heart and human adult liver are derived from normal tissues.

The condition of cultivation was similar to previous reports (22). The cells were grown as monolayers in 1000-ml culture bottles and were overlaid with 50 ml of growth medium which was made up of Hank's balanced salt solution containing 10% human serum, 10% glucose, 2% yeast extract, 1.4% $NaHCO_3$. To the solution was added an amount of penicillin and streptomycin to yield a final concentration of 100 units and 100 μg/ml, respectively. An antifungal agent (mycostatin 50 μg/ml, or fungizone 2 μg/ml) was added routinely once a month but was absent when the cells were planted for experimental study.

The cells were grown in a stationary state at 37C, and the growth medium was changed every other day. The inoculum of cells per each bottle was from 0.5 to 1.0×10^6, and by the time of harvesting, approximately 5 to 10×10^6 cells were in each bottle. After 7 days of growth, two to four bottles of cells were pooled in order to obtain a sufficient amount of material for analysis.

[1] Part of a thesis submitted to the Graduate School of the University of North Dakota in partial fulfillment for the degree of Doctor of Philosophy.

TABLE I
Biochemical Composition of HeLa and Human Heart Whole Cells in Culture

Cell line	No. exps.	Unit	DNA	RNA	Protein [a]	Phospho-lipids [b]
HeLa	12	μg/cell x 10^6	23.8 (1.0)[c]	53.0 (2.7)	310.2 (23.0)	96.7 (4.2)
	12	μg/μg DNA		2.22 (0.14)	13.22 (1.15)	3.07 (0.19)
Human heart	6	μg/μg DNA		2.08 (0.07)	15.99 (0.57)	2.88 (0.30)

[a] μg Protein-N x 6.25
[b] μg Lipid-P x 25
[c] The figures in parentheses indicate the standard deviation.

Cell Harvesting

After the growth medium was removed, the bottle was scraped with a rubber policeman, and the cells were washed off the bottle with ice-cold Hank's solution containing 11% glucose, 0.8% NaCl, and 0.04% KCl. The cell suspensions were pooled in a Potter-Elvehjem tissue grinder. An aliquot was removed for cell counting with a haemocytometer and the remainder was centrifuged in the cold at 700 $\times$ *g* for 5 min.

Cell Fractionation

The cells were taken up with 10 volumes (2 to 3 ml) of ice-cold 0.25 M sucrose solution containing 0.00018 M $CaCl_2$ and were homogenized with a Teflon pestle. In order to lower the concentration of suspension to minimize entrapment of particulates, the homogenate was brought to 9 ml with the sucrose-$CaCl_2$ solution. The suspension was centrifuged at 450 $\times$ *g* for 10 min. The precipitate was resuspended in sucrose-$CaCl_2$ solution and homogenization and centrifugation were repeated twice for the preparation of nuclear fraction. The pooled supernatant fractions were centrifuged at 10,000 $\times$ *g* for 15 min in a Servall Automatic Superspeed Refrigerated Centrifuge, type RC-2 to precipitate the mitochondria. The remaining supernatant was, in turn, centrifuged at 80,000 $\times$ *g* for 90 min in a Spinco (Beckman) Model L, Preparative Ultracentrifuge to precipitate the microsomes.

Biochemical and electron-microscopic examinations were carried out during the process of cell fractionation. The mitochondrial fraction did not have microsomal contamination as shown by the absence of glucose-6-phosphatase activity (21) and ribosome-like granules (free or membranes-attached). Microscopically the microsomal fraction contained solely smooth and rough surfaced endoplastic reticulum and free ribosomal particles. Microscopic and DNA analyses indicated the absence of nuclear contamination in both mitochondrial and microsomal fractions.

Extraction and Chromatographic Separation of Phospholipids

The lipids of the separated nuclear, mitochondria, and microsomal fractions were extracted once with 4 ml of 95% ethyl alcohol at 65C for 3 min and twice with boiling ethanol-ethyl ether, 2:1 (v/v) (21). The solvents were quickly removed in a rotary evap-

TABLE II
Biochemical Composition of Total Particulate Fractions from Various Mammalian Cells in Culture

Cell line	No. exps.	RNA μg / DNA μg	Protein μg / DNA μg	Phospholipid μg / DNA μg
HeLa	12	1.79 (0.05)[a]	8.36 (0.66)	2.38 (0.20)
KB	14	2.11 (0.09)	11.02 (0.61)	2.95 (0.25)
Human heart	3	2.02 (0.06)	13.99 (0.51)	2.10 (0.18)
Human liver	4	1.95 (0.05)	11.57 (0.34)	2.18 (0.13)

[a] The figures in parentheses indicate the standard deviation.

orator (Evapo-Mix, Rinco Instrument Co.) at 50C and 50 mm Hg in vacuum and the residue was lyophilized in a Freeze Dryer (VirTis Co., Inc.) overnight. The extract was further purified by dissolving in chloroform-methanol 1:1 (v/v). All lipid extracts were stored in a nitrogen atmosphere. The individual phospholipids were separated by chromatography on silicic acid-impregnated glass filter paper (3). The solvent system was diisobutyl ketone:acetic acid:water:benzene (160:50:8:7). The chromatograms were dried, stained with rhodamine 6G and the phospholipids were identified under ultraviolet light with synthetic standards and purified lipids as references (3). Recovery values of the individual phospholipids were similar to that reported by Cornatzer et al. (3).

A mixture of purified lipids containing as phosphorus, 2.93 μg of phosphatidyl inositol (Pierce Chemical Co.); 9.46 μg of sphingomyelin (Nutritional Biochemical Corp.); 6.82 μg of β,γ-dipalmitoyl-L-α-lecithin (Calbiochem), 0.53 μg of phosphatidyl serine (Pierce Chemical Co.), 7.70 μg of β,γ-dipalmitoyl-L-α-cephalin (Calbiochem) and 4.02 μg of polyglycerol phosphatide (Pierce Chemical Co.) was chromatogramed five different times on glass paper impregnated with silicic acid (3) with average recovery of the individual phospholipids of 97%, 107%, 110%, 92%, 88%, and 100% respectively.

Phospholipid Determination

The encircled spots on the dried, stained chromatograms were cut out and placed into 15 × 125 mm Pyrex test tubes, and were eluted with 5 ml of 3 N methanolic HCl for 50 min in a water bath at 63C. Marbles were placed over the mouths of the test tubes so as to prevent evaporation of the solvent. The elution of the phospholipids from the chromatograms was repeated with 5 ml of 2 N methanolic HCl. Three to four chromatogram spots of the same phospholipid were combined in order to obtain sufficient quantity of phosphorus for accurate measurement.

The quantitative determination of lipid phosphorus was performed by a modified colorimetric method of Shin (18) and Bartlett (2). After the pooled hydrolysate was evaporated to dryness, 1.0 ml of 18 N sulfuric acid was added. Digestion was carried out for 30 min on a medium gas flame. After the tube was cooled, 2 drops of H_2O_2 (30%) were added and the tube was heated for 30 min. Excess peroxide was removed by adding 2 drops of 5% urea solution and reheated for 15 min. The tube was cooled and 2.0 ml of distilled deionized water and 1 ml of 5% ammonium molybdate were added. After mixing, 0.25 ml of Fiske-SubbaRow reagent (18) was added and the solution was thoroughly mixed. The tube was placed in a water bath and was heated at 100C for 10 min. The tube was cooled and the optical density was determined at 800 mμ in a Cary Model 15 Recording Spectrophotometer (Applied Physics Corp.). A linear curve for lipid phosphorus (0.2–16.0 μg) was observed.

Nucleic Acids and Protein Determination

The dried residue after lipid extraction was treated with 5 ml of cold trichloroacetic acid (TCA) to extract the acid-soluble phosphorus fractions (17). Total nucleic acids were then extracted from the remaining residue with 3 ml of 5% TCA for 30 min at 90C according to the procedure of Logan et al. (10). The concentration of total nucleic acid was determined in a Beckman Model DU spectrophotometer at 268.5 mμ (10). DNA was determined by the use of the *p*-nitrophenylhydrazine method of Webb and Levy (24). RNA was obtained from the difference between total nucleic acid and DNA.

Protein-Nitrogen of the residue after lipid and nucleic acids extraction, was determined

TABLE III

Lipid–P/Protein–N (μg/mg) of the Subcellular Fractions from Various Mammalian Cells in Culture

Cell line	No. exps.	Nuclei	Mitochondria	Microsomes
HeLa	12	26.67 (2.99)[a]	107.78 (4.40)	59.19 (5.22)
KB	9	33.27 (3.84)	107.11 (5.85)	54.01 (2.89)
Human heart	5	45.70 (2.94)	74.70 (2.56)	39.45 (7.15)
Human liver	4	42.61 (1.78)	56.00 (3.80)	51.01 (1.01)

[a] The figures in parentheses indicate the standard deviation.

TABLE IV
The Chemical Composition of the Subcellular Fractions of HeLa and KB Cells in Culture

Cell line	No. exps.	Subcellular fraction	DNA μg / Cell	RNA μg / DNA μg	Protein μg / DNA μg	Phospholipid μg / DNA μg
HeLa	7	Nuclei	24.0 (2.5)[a]	0.29 (0.06)	3.54 (0.25)	0.38 (0.03)
	7	Mitochondria	..	0.38 (0.04)	3.11 (0.40)	1.33 (0.08)
	5	Microsomes	..	1.11 (0.11)	2.22 (0.10)	0.59 (0.05)
KB	10	Nuclei	24.7 (0.6)	0.59 (0.09)	4.60 (0.05)	0.64 (0.02)
	10	Mitochondria	..	0.47 (0.03)	4.52 (0.44)	1.95 (0.03)
	10	Microsomes	..	1.30 (0.12)	2.68 (0.14)	0.58 (0.04)

[a] The figures in parentheses indicate the standard deviation.

by means of the ultramicro-Kjeldahl method (16).

RESULTS

The chemical composition of whole cellular DNA, RNA, protein and phospholipid concentrations has been determined in HeLa and human heart cells (Table I). For comparison between the various cell lines, the concentrations of RNA, protein and phospholipids are related to the DNA. The concentration of cellular DNA is so constant (1) that it serves as a more accurate measuring parameter to which to relate the concentrations of other constituents. The concentrations of RNA, protein, and phospholipids (μg/μg of DNA) of the combined particulate fractions which include nuclei, mitochondria and microsomes, of HeLa, KB, human heart and human liver cells are presented in Table II. The concentration of various chemical components from various human tissue-culture cell lines are now

TABLE V
Phospholipid Composition of Subcellular Fractions of Various Human Tissue Culture Cell Lines
(Per Cent of Total Lipid–P)

Cell line	No. exps.	Subcellular fraction	Phosphatidyl inositol	Sphingo-myelin	Phosphatidyl choline	Unknown	Phosphatidyl serine	Phosphatidyl ethanolamine	Polyglycerol Phosphatide
HeLa	13	Nuclei	7.1 (5.8–9.3)	3.8 (2.7–5.4)	60.5 (59.8–62.2)	2.3 (2.1–2.8)	6.1 (5.7–6.4)	19.1 (16.8–21.8)	1.3 (0.6–2.5)
	13	Mitochondria	4.8 (4.1–6.0)	3.0 (2.7–4.4)	53.7 (50.9–55.4)	2.6 (2.3–3.3)	5.9 (5.4–6.3)	25.6 (23.2–30.5)	4.5 (3.2–6.0)
	13	Microsomes	6.7 (3.4–8.5)	3.2 (2.2–7.4)	65.1 (64.6–66.0)	1.9 (1.6–2.5)	6.7 (6.5–7.0)	16.3 (13.3–18.6)	0.4 (0.0–1.0)
KB	9	Nuclei	8.2 (8.0–10.3)	4.0 (2.2–4.8)	59.3 (57.1–60.0)	1.7 (1.1–2.2)	7.1 (5.4–7.3)	18.9 (17.3–20.0)	1.5 (1.0–1.9)
	9	Mitochondria	6.5 (5.4–7.7)	3.9 (3.1–5.9)	51.7 (51.0–52.4)	2.4 (2.0–2.9)	6.9 (6.2–7.6)	24.0 (21.4–25.0)	4.7 (4.2–5.3)
	9	Microsomes	8.3 (7.9–9.4)	4.1 (3.5–7.9)	62.0 (59.0–63.7)	2.2 (2.0–2.3)	7.8 (6.2–8.3)	15.1 (13.9–16.7)	0.5 (0.0–0.9)
Human heart	6	Nuclei	3.9 (3.4–4.5)	4.7 (4.2–5.3)	70.2 (66.6–74.0)	....a	....a	17.9 (15.3–19.0)	3.4 (1.7–4.6)
	6	Mitochondria	2.8 (1.7–3.4)	10.4 (8.6–12.4)	62.3 (59.4–63.6)	....	....	16.4 (15.5–18.2)	8.1 (7.3–10.9)
	6	Microsomes	2.7 (2.2–3.1)	11.4 (9.0–12.8)	71.3 (66.8–73.1)	....	....	14.6 (12.8–15.0)	.... (0.0–0.0)
Human liver	4	Nuclei	2.1 (1.3–2.6)	5.4 (4.8–6.6)	67.9 (65.3–69.4)	....	....	19.2 (17.4–21.2)	5.3 (4.3–6.5)
	4	Mitochondria	1.3 (0.6–1.6)	17.1 (16.4–18.2)	56.5 (54.5–58.5)	....	....	14.5 (13.2–16.4)	10.6 (9.2–11.6)
	4	Microsomes	1.4 (1.1–1.7)	16.0 (15.6–17.4)	63.5 (62.0–65.4)	....	....	19.1 (17.0–20.7)	....

The figures in parentheses indicate the range.
[a] The spots were not separated from phosphatidyl choline.

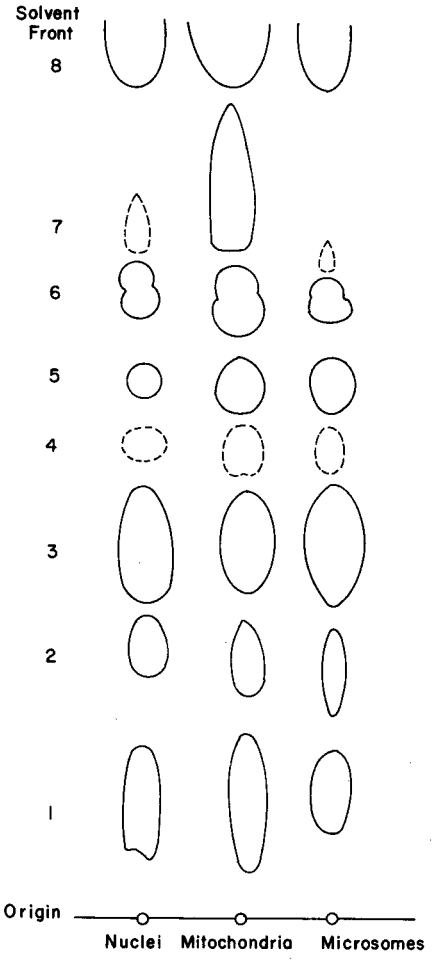

FIG. 1. Tracing of ascending chromatograms of the phospholipids of subcellular fractions of HeLa cell (10 to 20 μg per each spot). Chromatography was carried out at 20C for 6 hr on silicic acid-impregnated glass paper. The solvent system was diisobutyl ketone-acetic acid-water-benzene (160:50:7.5:7, v/v). The lipid spots were detected by staining with rhodamine 6G and observed while wet under ultraviolet light (366 mμ). The following colors were observed: spots 1, 4, 5, and 7 blue, and others yellow. Spot 1 corresponds to phosphatidyl inositol, 2 to sphingomyelin, 3 to phosphatidyl choline, 4 to unknown, 5 to phosphatidyl serine, 6 to phosphatidyl ethanolamine, 7 to polyglycerol phosphate (or cardiolipin) and 8 to neutral lipid.

comparable. The lipid P/Protein N ratio of the subcellular fractions prepared from HeLa, KB, human heart, and human liver cells are presented in Table III. The concentrations of DNA, RNA, proteins and phospholipids of nuclei, mitochondria, and microsomes of HeLa and KB cells are presented in Table IV. Over 50% of the cellular RNA is present in microsomal fractions whereas less than 20% of RNA is found in the nuclear or mitochondrial fraction of HeLa and KB cells. The cellular protein is approximately the same for nuclei and microsomal fractions. Mitochondrial fraction contains the highest phospholipid concentration as shown in Table IV. In order to determine whether the phospholipids were equally distributed in the subcellular fractions the individual phospholipids, phosphatidyl inositol, sphingomyelin, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and polyglycerol phosphatide (cardiolipin) were separated by chromatography on silicic acid impregnated glass filter paper. Fig. 1 shows the tracing of chromatograms of individual phospholipids of nuclei, mitochondria, and nuclei of HeLa cells (10 to 20 μg of phospholipid phosphorus). The individual phospholipids were identified with rhodamine 6G and an unknown fraction demonstrated a blue color with ultraviolet light. However, this fraction represented a small quantity of lipid phosphorus and could not be identified. The per cent composition of individual phospholipids of nuclei, mitochondria, and microsomes of HeLa, KB, human heart and liver cells are reported in Table V.

DISCUSSION

The RNA and protein content of HeLa cells is in agreement to that reported by Gifford (6). The DNA concentration of HeLa cells is similar to that reported by Massaab et al. (11). The DNA content of HeLa and KB cells is greater (14) than that of mammalian tissues (23) and is similar to Ehrlich ascite cells (8). The RNA, protein and phospholipid concentrations (expressed as μg/μg DNA) are similar for the four cell lines studied. The greatest RNA concentration and lowest protein concentration is found in the microsomes as compared to the other subcellular fractions.

Phospholipids are found in the membranes (7), mitochondria, nuclei, and microsomes (9,19,20) of mammalian tissues. Liver mitochondria of rats are composed of about 21–28% phospholipid on a dry weight basis, with the phospholipids accounting for about 90% of the total lipids present (19,21). Lipid P/Protein N of nuclei, mitochondria, and microsomes of HeLa, KB, human heart and liver cells were calculated since these subcellular structures are chiefly composed of protein and

phospholipids. The lipid P/Protein N content of mitochondria was greater than other subcellular fractions in all the tissue-culture cell lines studied. Human heart and liver cells of mitochondria contain a smaller lipid P/Protein N ratio than HeLa and KB cell lines. This observation is in agreement with that described by McCarl and Triebold (13), who have shown that phospholipids in HeLa, monkey heart, and chick embryo endothelium cells have a different rate of synthesis.

The per cent of total lipid phosphorus of the individual phospholipids of subcellular fractions of HeLa, KB, human heart, and liver cell lines have been determined. Phosphatidyl choline and phosphatidyl ethanolamine are the major phospholipids in the subcellular fractions of the tissue-culture cell lines studied. This observation is similar to concentrations observed for the subcellular fraction of liver, kidney and heart of the rat (15). The per cent of total lipid-P composition of phosphatidyl choline is greater in microsomes than in other subcellular fractions of all cell lines except the nuclear fraction of human liver cell. Correspondingly, the mitochondrial fraction from all the tissue-culture cell lines contains the greatest amount of phosphatidyl ethanolamine except in the human liver and heart cells and contains the lowest concentration of phosphatidyl inositol. This observation is in accordance with the finding in normal mammalian tissues (4,12,20). Strickland and Benson (20) observed 30% of the total lipid phosphorus was phosphatidyl ethanolamine in mitochondria of rat liver compared to 18% in microsomes; Getz et al. (4) found 42% in mitochondria and 27% in microsomes of rat liver, and Marinetti et al. (12) observed in pig heart that the mitochondria contain 25.3% and 21.2% in microsomes. It is apparent from the data of Table V that the mitochondrial fraction from all the cell lines studied contains less phosphatidyl choline and phosphatidyl inositol in relation to phosphatidyl ethanolamine than do microsomes or nuclear fractions. Strickland and Benson (20) observed 68% of total lipid phosphorus was phosphatidyl choline in microsomes of rat liver compared to 49% in mitochondria; Getz et al. (4) found 62.6% in microsomes and 38.6% in mitochondria of rat liver, and Marinetti et al. (12) observed in pig heart 41.8% in microsomes and 36.2% in mitochondria. The ratio of phosphatidyl choline/phosphatidyl ethanolamine is 4.0 in microsomes and only 2.1 in mitochondria of HeLa cells. The values are 4.1 and 2.15 respectively for KB cells. Similar values have been reported in subcellular fractions of rat liver (5). Polyglycerol phosphatide occurs chiefly in the mitochondria of tissue-culture cells as in mammalian tissues (12). The per cent composition of polyglycerol phosphatide appears to be higher in human heart and liver than in HeLa and KB cells.

Thus, the four different lines of cultured cells of human tissue contain the major phospholipids which are found in normal mammalian tissues. It is apparent from the data that there is no substantial difference in the per cent of total lipid phosphorus of human tissue-culture cells when compared to mammalian tissues (4,12,20).

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Isolation of a Trimer of α-Tocopherol from Mammalian Liver

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ABSTRACT

Evidence is presented for the formation in mammalian liver of a trimeric metabolite of α-tocopherol. This compound has been shown to be identical to a trimer produced by oxidation of α-tocopherol with alkaline $K_3Fe(CN)_6$. In addition, confirmation was obtained for the occurrence in vivo of a dimeric metabolite reported previously. These compounds, together with tocopheryl-*p*-quinone, are postulated to arise from reactions with lipid-free radicals or peroxides in the course of the antioxidant action of vitamin E.

INTRODUCTION

IN EARLIER INVESTIGATIONS of the metabolism of *d*-α-tocopherol-5-methyl-C^{14} carried out in this laboratory, it was observed that this vitamin undergoes partial conversion in rat tissues to α-tocopheryl-*p*-quinone and a dimer (1,2). The dimer was shown to be identical to a compound obtained by oxidation of α-tocopherol with alkaline $K_3Fe(CN)_6$ (3). In the experiments described here, evidence has been obtained for the formation in vivo of a trimer of α-tocopherol and confirmation was obtained for the natural occurrence of the dimer. The trimer is the unidentified metabolite reported in a preliminary abstract (4) and cannot be differentiated from the synthetic oxidation product of α-tocopherol previously designated Compound II (2).

EXPERIMENTAL

The relative susceptibility of α-tocopherol to oxidation under conditions commonly used for the isolation of unsaponifiable lipids makes it mandatory that particular precautions be taken against the occurrence of isolation artifacts. This problem is emphasized by the finding that there is an analogy between the products of α-tocopherol oxidation formed in vivo, those obtained by chemical oxidation with certain inorganic ions and those generated in a milieu of peroxidizing unsaturated lipids (unpublished results). Consequently, in the present study the natural occurrence of the metabolites in question was investigated by several different isolation procedures and in conjunction with recovery studies on *d*-α-tocopherol-C^{14} internal standards.

Rats were injected intraperitoneally with 1 mg of *d*-α-tocopherol-5-methyl-C^{14} (specific activity 1.5 μc/mg) which was prepared from the succinate ester (obtained from Distillation Products Industries, Rochester, N. Y.) by reductive cleavage with $LiAlH_4$. The reduction product was checked for purity and prepared for administration as described previously (3). Rabbits were injected intravenously by ear vein with 2 mg of the same material. Rats were killed 48 hr after injection, rabbits after 24 hr; the livers were processed by one of the procedures outlined below.

Procedure I

Livers taken from 4 injected rats deprived of vitamin E for 4 weeks were minced on a chilled watch glass, quick-frozen over dry ice and lyophilized overnight. Three livers from comparable uninjected animals were treated similarly, except that pure *d*-α-tocopherol-C^{14} (183×10^3 dpm in 50 μl of emulsion used for injection) was mixed thoroughly with the minced tissue before freezing. This amount of tocopherol-C^{14} was based on the general observation that after 48 hr about 5% of the dose was ordinarily recovered in the liver; the actual recovery for the 4 injected animals averaged 4.8%.

The dry residue was extracted twice by shaking for 15 min with 50 ml of double-distilled petroleum ether (Skellysolve F, bp 30C–60C), then was transferred to a filter paper and washed with an additional 150 ml of the same solvent. After evaporation of the solvent under N_2 on a water bath the residue was dissolved in 15 ml of acetone and the bulk of the lipids was removed by crystallization at −70C as described previously (1). The material remaining after filtering off the lipids and evaporating the solvent from the filtrate (18–20 mg) was chromatographed on 8×17.5 in. sheets of glass paper (2 sheets per sample) with 90% acetonitrile in H_2O as developing solvent. In this system pure α-tocopherol and α-tocopheryl quinone move to the solvent front whereas the tocopherol dimer and trimer (2) remain at the origin. In the

presence of liver lipids, however, some tocopherol and quinone are also retained at the origin. A band 4 cm wide was taken from the origin, cut into small pieces and shaken for 10 min with 50 ml of petroleum ether. The paper then was washed 5 more times with 25 ml of solvent and the combined filtrates were evaporated under N_2 to 5 ml.

This solution was mixed with 3–4 mg each of carrier α-tocopherol, dimer and trimer (2). This mixture was applied to a 1×16 cm column of petroleum ether-washed Bio-rad neutral alumina (6% water added) which was developed with increasing proportions of peroxide-free diethyl ether in petroleum ether. Fifty-one to sixty 5-ml fractions were collected, of which 1 ml was used for the determination of radioactivity by liquid scintillation spectrometry and 4 ml were used to determine the optical density in isooctane at the appropriate absorption maximum for each carrier.

Procedure II

An attempt was made to confirm the evidence obtained by Procedure I for the natural occurrence of the tocopherol trimer with another species and an isolation method based on paper and thin-layer chromatography. Three normal growing rabbits were injected with labeled tocopherol and the livers from two others were used as controls. The procedures for lyophilization, crystallization of lipids (twice) and chromatography on glass paper (3 sheets per sample) were the same as those outlined for Procedure I. The band at the origin of the glass paper chromatograms was eluted with 100 ml of petroleum ether and purified on thin layers of Silica Gel G with 90% acetonitrile in H_2O as the mobile phase. The material at the origin was eluted with ether, and again purified on glass paper; an aliquot was then chromatographed on paraffin-coated paper (3) with 75% ethanol. A scanogram revealed the presence of a single peak at the origin, consistent with the presence of dimer and trimer, and showed no peaks for tocopherol or tocopheryl quinone. To separate the dimer and trimer the samples were chromatographed on thin layers of silica gel with benzene-hexane (45:55). Four-centimeter bands opposite the standard dimer (R_f 0.25) and trimer (R_f 0.80) were eluted with ether and purified once more on silica gel using a system (2.5% methanol in benzene) in which the dimer moves farther from the origin (R_f 0.80).

As a further check on the purity of the labeled compounds isolated by Procedure II, they were pooled into two samples representing the final dimer and trimer fractions, appropriate carrier compounds were added and the mixtures were rechromatographed on alumina columns as described for Procedure I. The dimer bands from the two control samples were chromatographed individually; the trimer bands contained too few counts for further purification.

Carrier crystallization was used to confirm the evidence obtained by cochromatography (Fig. 1 and 4) that one of the labeled isolates was a trimer of α-tocopherol. Molecular weight estimations made on the synthetic carrier with a Mechrolab vapor pressure osmometer yielded values of 1294 (in benzene) and 1211 (in $CHCl_3$) (theoretical molecular weight for a trimer of α-tocopherol, approximately 1292). To 102 mg of this trimer was added 2.1×10^4 dpm of labeled material obtained by pooling the 2% ether fractions from the injected rats (Fig. 1) and the counts recovered in the analogous fractions from C^{14}-tocopherol-injected rabbits (Fig. 4). The mixture was reduced with $LiAlH_4$ (3) and esterified with *p*-nitrobenzoyl chloride in the presence of butyllithium as described by Brown et al. (6). After dissolving the combined sample in 2 ml of petroleum ether, 1 ml of 15% *n*-butyllithium in hexane was added and the mixture was refluxed for 30 min. Then 65 mg of *p*-nitrobenzoyl chloride in 1 ml ether were added carefully with stirring, and refluxing was continued for an additional 2 hr. The mixture was cooled, diluted with H_2O and extracted 3 times with ether. After purification on a Bio-rad alumina column (6% H_2O added), the product (52 mg) was shown to be fully esterified by infrared analysis, and was crystallized from petroleum ether at −15C.

Procedure III

The fact that it was found necessary to use column chromatography in Procedure II for the final isolation of radiochemically pure metabolites suggested that they could be obtained without preliminary purification by direct alumina chromatography of the liver extracts. The livers from 6 normal 300-g rats were dried with Na_2SO_4 and extracted individually overnight by shaking with acetone (3). After crystallizing and filtering off most of the lipids at −70C, the filtrate residues were combined, applied to a 2 cm $\times$40 cm column of neutral alumina (6% water added), and developed with ether-petroleum ether mixtures. The starting material contained 3.76×10^6 dpm

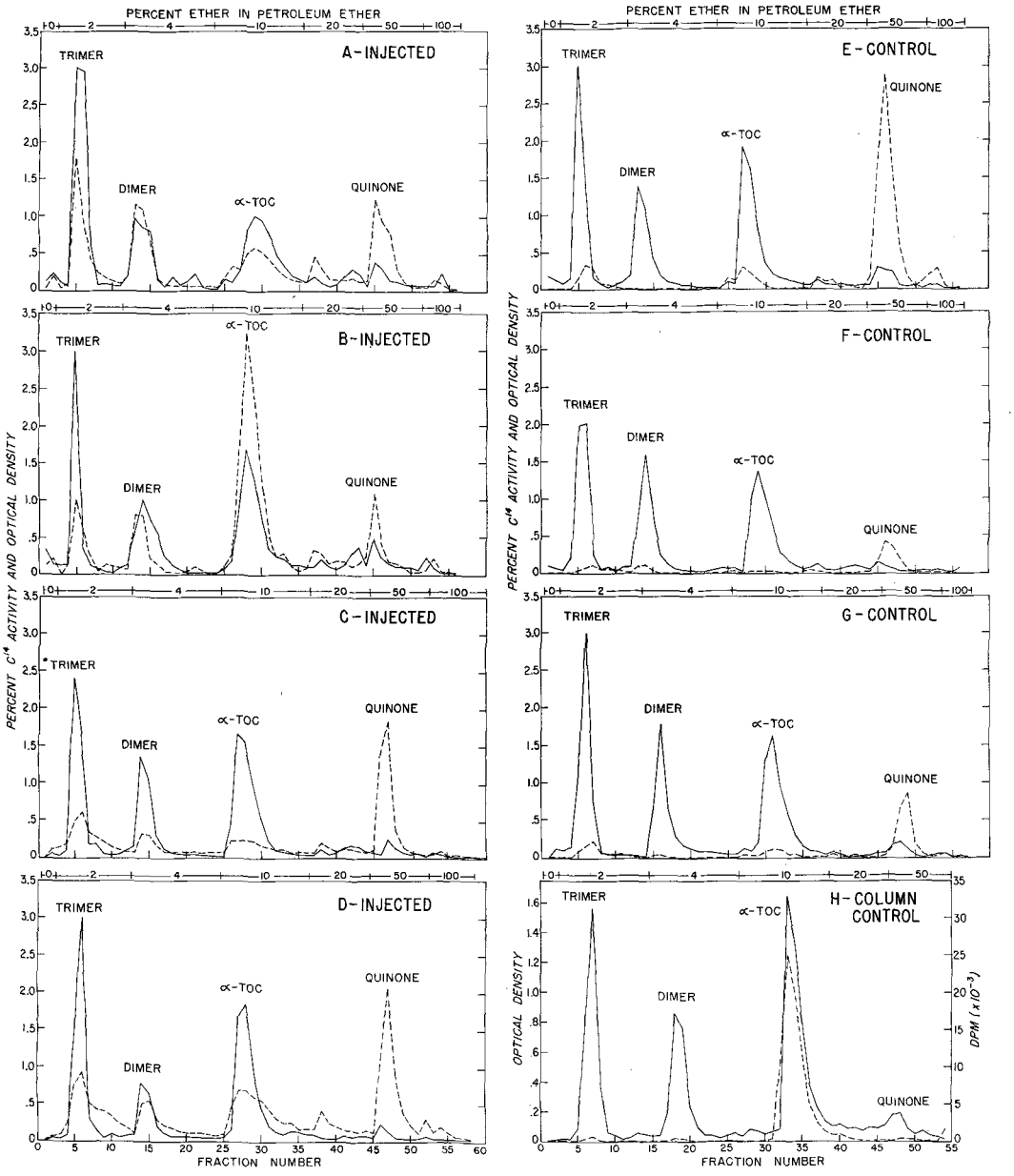

FIG. 1. Elution of labeled rat liver compounds from neutral alumina columns after preliminary purification by glass paper chromatography (Procedure I). % C^{14} activity (------) = (dpm in fraction /dpm in liver extract) 100. O.D. for carrier compounds (——) was recorded at their respective absorption maxima: 298 mμ for α-tocopherol, 300 mμ for dimer and 295 mμ for trimer. Nonspecific absorption between carrier peaks was recorded at 295 mμ.

(7.0% of the dose). Pure α-tocopherol-C^{14} equivalent to 5.0% of the dose was added to 2 control liver homogenates which were then carried through the isolation procedure simultaneously.

Procedure IV

The livers from 3 vitamin E-deficient rats which had been injected with α-tocopherol-C^{14} were saponified with ethanolic KOH in the presence of pyrogallol as described elsewhere

(5). The unsaponifiable fraction was applied in a minimum volume of petroleum ether to the top of a 1.75 cm × 20 column of glass paper which had been shredded with a Waring blendor in 65% aqueous ethanol. Starting with this solvent, the sample was washed with increasing percentages of ethanol in water. The main C^{14} band (3.1×10^5 dpm) was eluted with 80% alcohol. The residue from this fraction was chromatographed on sheets of paraffin-coated paper with 75% ethanol as mobile phase, in which system the dimer and trimer remain at the origin and α-tocopherol migrates to an R_f of 0.25. Extraction of the origin with absolute ethanol yielded material containing 26.5×10^2 dpm which was further purified by chromatography on a neutral alumina column as described above.

RESULTS

Procedure I

The C^{14} elution profiles for the 4 injected and 3 control animals are illustrated individually in Fig. 1, together with that for a control in which a pure sample of α-tocopherol-C^{14} was chromatographed on a column of alumina by the same procedure. Peaks of radioactivity coinciding with those for each of the three carriers are in evidence, plus an additional peak in the 50% ether fraction.

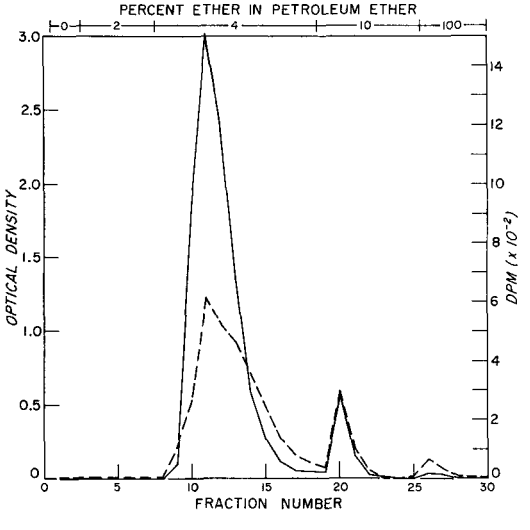

FIG. 2. Cochromatography on a neutral alumina column of the *p*-nitrobenzoic acid esters of a C^{14}-labeled metabolite of vitamin E isolated from rat and rabbit liver (------) and of a trimer synthesized by $K_3Fe(CN)_6$ oxidation of α-tocopherol. The O. D. (——) was recorded at the absorption maximum of the ester (254 mμ).

The C^{14} peaks for the control livers corresponding to the carrier trimer and dimer are much smaller than are those for the injected animals, and in two controls (Fig. 1E and 1G) the counts in the dimer fractions were negligible. The data indicate that although small amounts of dimer and trimer probably were formed as artifacts in the course of this procedure, the labeled compounds derived from the livers of the injected rats were primarily of in vivo origin. Expressed as percentages of the total counts extracted from the livers of the injected animals, the counts eluted in the dimer and trimer fractions averaged 2.1% and 3.0%, respectively.

α-Tocopherol Fractions. The identification of the labeled compound eluted with 10% ether (Fig. 1) as α-tocopherol was confirmed by thin-layer chromatography and by cochromatography on paraffin-coated, silicone-coated and $ZnCO_3$-impregnated paper as described elsewhere (3). The variability in the amount of α-tocopherol-C^{14} eluted from the column undoubtedly is due mainly to differences in the amounts of this compound retained by the lipid at the origin of the glass paper sheets.

Dimer Fractions. The counts in the 4% ether fractions have been indentified previously with a tocopherol dimer by cochromatography and by carrier crystallization (2,3).

Tocopheryl p-Quinone Fractions. The counts in the 50% ether fractions (Fig. 1) evidently reflected mainly or entirely artifacts, since a similar proportion of the total counts extracted from the control livers also was recovered in this eluate. Column chromatography per se was not an important course of artifact formation, as pure α-tocopherol-C^{14} yielded negligible amounts of the *p*-quinone when chromatographed on alumina (Fig. 1H); however, a small peak indicative of quinone formation from the tocopherol carrier is discernible in most of the elution profiles. Since it has been shown repeatedly that α-tocopherol-C^{14} is the predominant labeled compound present in the liver extracts, the presence of relatively large numbers of counts in the 50% ether fractions can best be explained by assuming that they represent compounds formed by oxidation of lipid-bound tocopherol at the origin of the glass paper.

The residue from the pooled 50% ether fractions from the injected rats was rechromatographed on alumina with α-tocopherol and tocopheryl *p*-quinone carriers. Development of the column with ether-petroleum ether mixtures yielded two peaks of radioactivity, one

in the 30% ether fractions which coincided with the quinone carrier and the other in the 40% ether fractions. The identification of the first compound as the *p*-quinone was confirmed by reducing it to C^{14}-α-tocopherol with $SnCl_2$ in dioxane-HCl (1). The substance eluted with 40% ether remains unidentified, but since it was obtained in comparable amounts when the above procedure was applied to the corresponding 50% ether fractions from the control columns (Fig. 1), it apparently was present as an artifact.

Trimer Fractions. The common identity of the labeled compound eluted with 2% ether and the trimer carrier, which is indicated by their behavior on alumina (Fig. 1 and 4), was confirmed by the results of carrier crystallization. Recrystallization yielded products which exhibited the following specific activities (dpm/mg): 57, 31, 20, 18, 17. The final crystals, which were liquid at room temperature, were rechromatographed on alumina (6% H_2O added). The main peak of radioactivity coincided with that of the carrier ester (Fig. 2) which was estimated by the extinction at its absorption maximum (254 mμ). The small coinciding peaks in the 10% ether fractions probably represent partially hydrolyzed products, the formation of which provided additional confirmation of the common identity of the radioactive and carrier compounds.

Procedure II

The results of this experiment, depicted in Fig. 3 and 4, demonstrate that despite extensive purification by paper and thin-layer chromatography, the "dimer fraction" was still heavily contaminated with α-tocopherol, tocopheryl quinone and a compound which was eluted with 20% ether (Fig. 3A). The identities of the first two compounds were confirmed by paper chromatography by reduction of the quinone to α-tocopherol-C^{14} with $SnCl_2$. Whereas a distinct dimer-C^{14} peak is evident

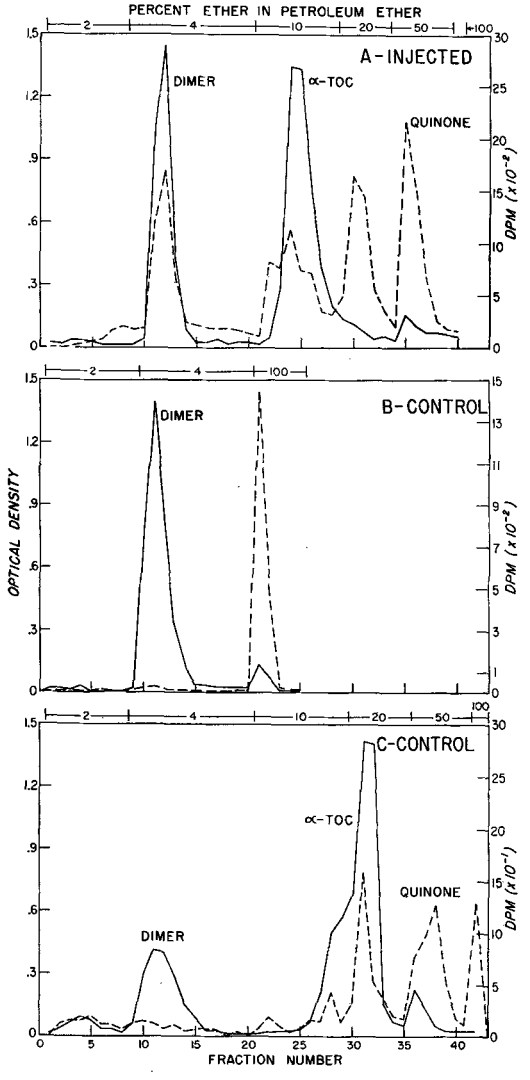

FIG. 3. Chromatography of the "dimer fractions" from rabbit liver on neutral alumina columns after preliminary purification by glass paper and thin-layer chromatography (Procedure II). The lines represent C^{14} activity (------) and O.D. of the carrier compounds (—).

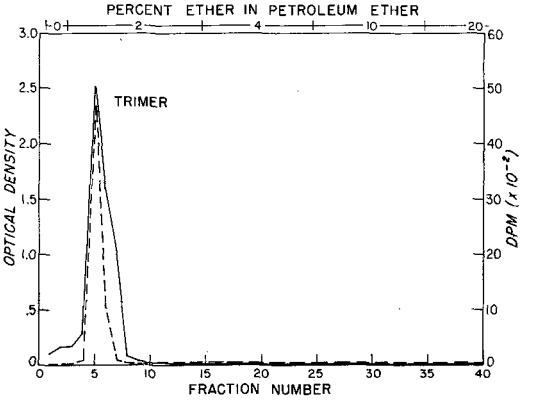

FIG. 4. Cochromatography on a neutral alumina column of the C^{14} counts (------) in the "trimer fractions" isolated from rat liver (Procedure II) and of a trimer synthesized by $K_3Fe(CN)_6$ oxidation of α-tocopherol. The O. D. (——) was recorded at the absorption maximum of the trimer (295 mμ).

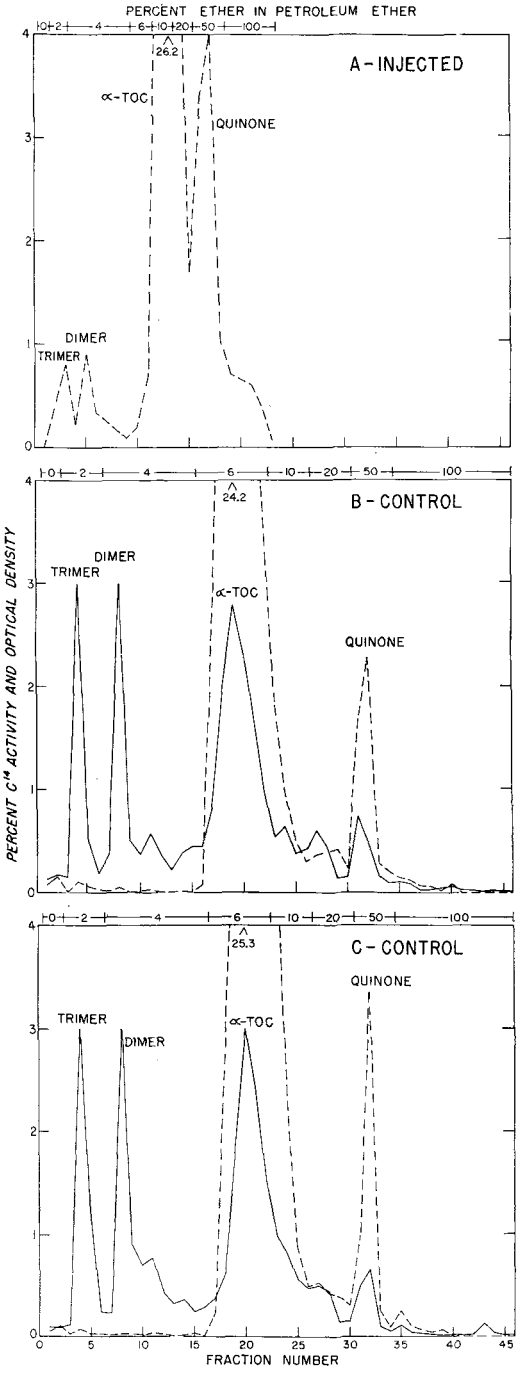

FIG. 5. Elution of labeled rat liver compounds from neutral alumina columns (Procedure III). % C^{14} activity (------) and the O. D. of the carrier compounds (——) were calculated as for Fig. 1. Eluate volume used for A (100 ml) was twice that used for B and C.

in the case of the extracts derived from the injected rabbits, the elution profiles for the control livers (Fig. 3B and 3C) show negligible activity in the dimer region and a preponderance of counts in other fractions. After collecting the dimer fractions from the first control sample the counts remaining on the column were eluted with 100% ether (Fig. 3B); more detailed chromatography of the counts from the second control showed that these remaining counts were eluted mainly in the α-tocopherol and quinone fractions (Fig. 3C).

In contrast to the dimer, the trimer-C^{14} isolated by paper and thin-layer chromatography was found to be radiochemically pure (Fig. 4). This is explainable by the fact that the trimer moved farther from the origin on the thin-layer chromatograms, whereas the dimer was incompletely separated from traces of lipid which evidently bound small amounts of labeled tocopherol and its *p*-quinone at the origin.

Procedure III

The results of direct column chromatography of the liver extracts from the C^{14}-tocopherol-injected rats are illustrated in Fig. 5. Four C^{14} peaks are apparent, representing the trimer, dimer, α-tocopherol and the *p*-quinone, respectively. As no preliminary step for the removal of α-tocopherol-C^{14} was used in this procedure, the extracts contained a predominance of this compound. The elution profiles for the controls show a distinct peak for the quinone (50% ether fractions) in each case, but negligible counts in the dimer fractions and only questionable activity attributable to the trimer. Of the total counts present in the acetone extracts 2.0% and 1.2%, respectively, were recovered in the dimer and trimer peaks from the injected animals and an average of 0.1 and 0.2%, respectively, from the control livers.

Fig. 5 also indicates that significant amounts of tocopheryl quinone were formed during the procedure. Of the total counts in the liver extracts, 8.5% were eluted in the quinone fractions from the injected animals compared to 4.2% and 4.7% from the two controls.

Procedure IV

Chromatography of the unsaponifiable liver fractions on alumina yielded a C^{14} peak in the 4% ether fractions which contained 16.3×10^2 dpm or 5.3% of the total counts. The residue from these fractions was combined with 300 mg of synthetic dimer, dissolved in 5 ml of

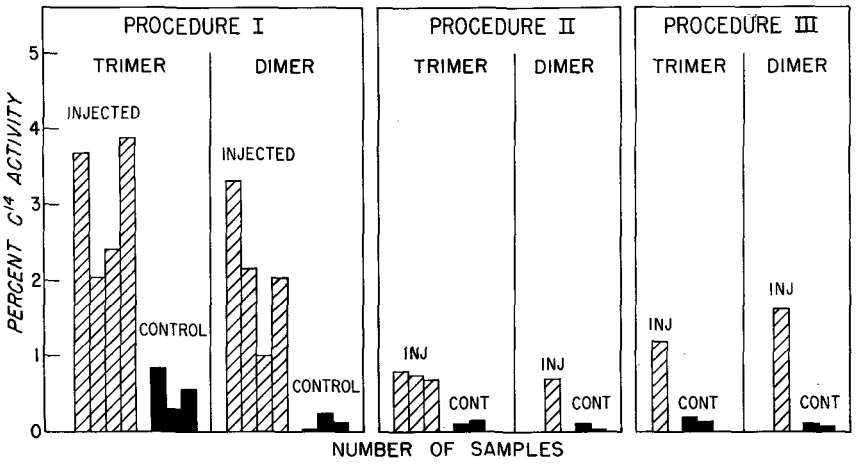

FIG. 6. Comparison of the relative proportions of counts associable with C^{14}-labeled dimer and trimer isolated from the livers of the injected animals and from the controls. % C^{14} activity = (total dpm in dimer or trimer fraction from alumina columns/total dpm in liver extract) 100.

acetone and cooled to −70C. Six crops of fine precipitate were collected by reprecipitation, 3 mg of each being taken for dry combustion. The $C^{14}O_2$ produced was collected on a high vacuum line (1). The specific activity values for the last 5 precipitates (dpm/mg) were as follows: 66.4. 43.0, 19.5, 18.0, 18.0. This experiment served to confirm the presence of the dimer by an extraction procedure greatly different from that employed previously (3).

DISCUSSION

The foregoing experiments provided consistent evidence for the formation in vivo of a trimer, as well as of a dimer, of α-tocopherol. In some cases the controls indicated that small amounts of these compounds also were formed as artifacts of the procedure; in others the amounts, if any, were not distinguishable from background counts. The counts recovered in the dimer and trimer fractions from the controls by the procedures described represented 5.6—8.0% and 14.0—19.3%, respectively, of those recovered in the corresponding fractions from the injected animals. As background radioactivity represented a significant portion of the total counts present in the fractions derived from the control samples, the above values consequently overestimate the actual extent of artifact formation. A summary of the relative yields of counts associable with the dimer and trimer is presented graphically in Fig. 6. Quantitatively, the *p*-quinone is a much more significant product of artifact formation during chromatography than is either of the other compounds.

The recovery of relatively uniform yields of labeled dimer and trimer by the different procedures employed also militates against the possibility that these compounds arose exclusively as isolation artifacts. Saponification of rat liver tissue in the presence of pure C^{14}-α-tocopherol according to Procedure IV failed to generate detectable amounts of C^{14}-dimer or trimer. The mincing operation used in Procedures I and II was carried out in about 30 sec over ice and therefore is an unlikely cause of artifact formation. Thin-layer chromatography of vitamin E in the presence of unsaturated lipid was the most likely source of spurious oxidation products.

The present findings support those reported previously relative to the occurrence in vivo of a tocopherol dimer (2,3), with the exception that in this study considerably smaller proportions of the metabolite were found in the liver extracts. The earlier estimates may have been the result of prolonged depletion of the animals on a vitamin E-deficient diet (1 year), or to incomplete separation of lipid-bound α-tocopherol-C^{14} remaining at the origin on silica gel, as suggested by Plack and Bieri (7). The present results favor the latter explanation. Confirmation of the presence in rat liver of a vitamin E metabolite, apparently either the dimer or trimer, has been reported recently by Mellors and Barnes (8).

The results of these and previous experiments (2,3) indicate that the vitamin E metabolite detected by Alaupovic and co-workers (9,10) and designated "Compound O" was a mixture of dimer and trimer. Apart from the possible occurrence of a minor metabolite in the 20% ether fractions from alumina (Fig. 3) which may be attributable to the presence of reduced dimer (unpublished results) the dimer, trimer and *p*-quinone were the only metabolites detected in this study. No evidence was obtained for the presence of vitamin $E_{2(50)}$, a proposed active form of tocopherol in the body (11), or for the urinary metabolite of Simon and co-workers (12).

The dimer (13), trimer (unpublished results) and *p*-quinone of α-tocopherol have no significant biological activity with respect to the prevention or cure of vitamin E deficiency in animals. They appear to represent end products of antioxidant reactions in vivo between tocopherol and lipid-free radicals or peroxides. In support of this conclusion the air oxidation of linoleate in the presence of vitamin E has been observed to result in the formation of similar tocopherol oxidation products (unpublished results). This observation, together with the demonstration that certain synthetic antioxidants can substitute for vitamin E in respect to the treatment of tocopherol deficiency diseases, is consistent with the view that the function of this vitamin in mammalian systems is associated with its antioxidant properties. The evidence in support of this conclusion has been reviewed elsewhere (14, 15). Although the dimer and trimer apparently arise from reactions with peroxidized lipids, their presence in the tissues of undepleted animals indicates that they are nevertheless normal metabolites of vitamin E.

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Phospholipids of the Sea Anemone: Quantitative Distribution; Absence of Carbon-Phosphorus Linkages in Glycerol Phospholipids; Structural Elucidation of Ceramide Aminoethylphosphonate

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ABSTRACT

The phospholipid composition of the sea anemone *(Anthopleura elegantissima)* was determined by quantitative thin-layer chromatography (TLC). Phosphonic acids were not detected in phosphatidyl choline, phosphatidyl ethanolamine, or phosphatidyl serine following isolation and hydrolysis. The structure of ceramide aminoethylphosphonate, a phosphonolipid, was elucidated by hydrolysis and oxidative degradation followed by characterization of the products by TLC and gas-liquid chromatography. The long-chain base was shown to be sphingosine with the 2-aminoethylphosphonic acid group attached through the primary hydroxyl group at carbon one. Ceramide aminoethylphosphonate is therefore 1-(O-phosphonoethylamine)-N-acyl sphingosine.

INTRODUCTION

THE RESULTS OF EARLY investigations of lipids of the sea anemone were somewhat conflicting (1,2) and later qualitative studies by paper and thin-layer chromatography indicated a different lipid composition (3–5), although no unique lipid classes were detected. Following demonstration of 2-aminoethylphosphonic acid as an hydrolysis product of a proteolipid preparation (7) and a crude lipid extract of the sea anemone (8), Rouser et al. (6), using column and paper chromatography, reported some quantitative values and isolated and partially characterized a new lipid, ceramide aminoethylphosphonate (CAEP).[2]

In this report we present: 1) the distribution of phospholipids of the sea anemone determined by quantitative TLC; 2) evidence for the absence of the carbon-phosphorus linkage in phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine; 3) data elucidating the structure of CAEP.

MATERIALS AND METHODS

Quantitative TLC

The procedure of Rouser et al. (9) was used with chloroform/methanol/28% aqueous ammonia 65/35/5 followed by chloroform/acetone/methanol/acetic acid/water 5/2/1/1/0.5 for two-dimensional TLC. The phosphorus content of spots was determined after aspiration.

Isolation and Identification of CAEP, PC, PE, and PS

Part of the original CAEP preparation isolated by Rouser et al. (6) was used for hydrolysis and demonstration of sphingosine by gas chromatography of its trimethylsilyl ether. A larger amount, used for oxidative degradation, was isolated essentially as previously described (6) following extraction with chloroform/methanol 2/1. The crude chloroform/methanol extract was freed of water-soluble nonlipid contaminants by Sephadex column chromatography (10). A mixture of CAEP and phosphatidyl ethanolamine was recovered by diethylaminoethyl (DEAE) cellulose column chromatography and CAEP and phosphatidyl ethanolamine were separated by silicic acid column chromatography. Phosphatidyl choline was obtained by DEAE column chromatography by elution with chloroform/methanol 9/1 following elution of less polar lipids with chloroform. Pure phosphatidyl serine (11) was eluted from a DEAE column with glacial acetic acid (6).

The pure lipids were identified by column chromatographic properties on DEAE cellulose and silicic acid, by two-dimensional TLC (12) and infrared spectroscopy (6) of the intact lipids, and acid hydrolysis followed by identification of products by paper and TLC and infrared spectroscopy (6).

Hydrolysis and Chromatography for Demonstration of Phosphonic Acids

Hydrolysis was performed under conditions

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[2] Abbreviations: NL, neutral lipid; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PS, phosphatidyl serine; PI, phosphatidyl inositol; DPG, diphosphatidyl glycerol (cardiolipin); PA, phosphatidic acid; CAEP, ceramide aminoethylphosphonate.

that cleave phosphate esters and leave phosphonic acids intact. Phospholipid (5 mg) was suspended in 2 ml of 2.5 N hydrochloric acid in a sealed tube and heated at 125C for 100 hr. The hydrolysate was evaporated to dryness, the residue suspended in 3 ml of water, filtered and concentrated to 0.1 ml. Part of the solution was used for paper chromatographic separation on Whatman 3MM paper with the upper phase obtained from the mixture of 1-butanol/acetic acid/water 4/1/5. Ninhydrin (6) and phosphate reagents (20) were used to detect spots. Ethanolamine, 2-aminoethylphosphonic acid, ethanolamine-O-phosphate, choline, phosphoryl choline, serine and alanylphosphonate were applied as standards. Paper electrophoresis in 1 N formic acid for separation of serine and alanylphosphonate was kindly performed by James Kittredge.

Determination of CAEP Fatty Acid and Long Chain Base Composition

Five milligrams of the CAEP preparation isolated in 1961 and stored in chloroform/methanol 2/1 at —20C was suspended in 2.5 ml of aqueous methanolic hydrochloric acid (8.9 ml 12 N acid, 9.4 ml water diluted to 100 ml with redistilled methanol) in a test tube. The solution was flushed with nitrogen and the tube sealed and heated at 70C for 18 hr (14). The tube was cooled, opened and solvent removed by evaporation under a stream of nitrogen. The residue was dissolved in a small volume of methanol and an aliquot used for TLC examination with chloroform/methanol/water 65/25/4 as solvent. A synthetic sample of sphingosine (Yeda Research and Development Company, Ltd., P.O. Box 26, Rehovoth, Israel) was used as a standard. The remainder of the methanolic solution was separated into fatty acid methyl ester and sphingosine fractions by the silicic acid column chromatographic method of Gaver and Sweeley (14). The methyl ester fraction was dissolved in hexane and fatty acids separated by GLC on ECNSS-S and SE-30 columns (15). Fatty acids were identified by comparison with pure standards and from a plot of log retention time versus carbon number. The relative amounts of fatty acids were estimated by determining the area percentage for each peak. The trimethylsilyl ether of sphingosine was prepared and its retention time relative to methyl stearate determined as described by Gaver and Sweeley (14).

A second 5-mg aliquot of CAEP was hydrolyzed by suspension in 2 ml of 2 N hydrochloric acid in a sealed tube and heating at 100C for 12 hr. The free fatty acids were extracted into diethyl ether and the solution washed with 0.1 N hydrochloric acid and dried over anhydrous sodium sulfate. After evaporation of the ether under a stream of nitrogen, the acids were suspended in 1 N methanolic sulfuric acid and heated in a sealed tube at 100C for 2 hr. The methyl esters were extracted into hexane and separated by GLC as described above.

Oxidative Degradation of CAEP and Identification of Myristic Acid and Serine as Final Products

The procedure previously described (16) was used with minor modification. With this procedure myristic acid and serine are obtained if the long chain base is sphingosine and the polar group is attached to carbon one, the hydroxyl group at carbon three being free (see Diagram 1). The reaction sequence is the same as for sphingomyelin (16).

A 20-mg sample of CAEP was used. Modifications of the original procedure were as follows. The aldehyde obtained from periodate oxidation was extracted with ether. The solvent was removed under a stream of nitrogen and the residue dissolved in 3 ml of glacial acetic acid containing 0.3 ml of a saturated aqueous solution of chromium trioxide. Oxidation was carried out in a glass-stoppered tube at 50C for 3 hr, 3 ml of water added and the solution extracted with 2 ml of *n*-hexane three times. The combined extracts were evaporated to dryness, the fatty acids methylated with 1 N methanolic sulfuric acid, the methyl esters extracted into hexane and identified by GLC as described above.

The ethanolic hydrochloric acid solution obtained after periodate oxidation contained the nitrogenous portion of the original CAEP molecule. Solvent was removed under a stream of nitrogen, the residue dissolved in 4 ml of 1 N hydrochloric acid and applied to a 2 (I.D.) × 20 cm Dowex 50 column in the hydrogen form. Salts were eluted with 3 column volumes of water and amino acid eluted with 5 column volumes of 1.5 N ammonium hydroxide. The ammonium hydroxide solution was evaporated to dryness, dissolved in 0.2 ml of water and serine identified using unactivated silica gel-magnesium silicate (9/1, w/w) as adsorbent and 1-butanol/formic acid/water 70/15/15, acetone/water 40/60 and phenol/water/methanol 70/20/10 as solvents. The oxidation product was compared with an authentic sample of serine. Spots were visualized after spraying with a solution of ninhydrin (1 mg/ml) in 1-butanol.

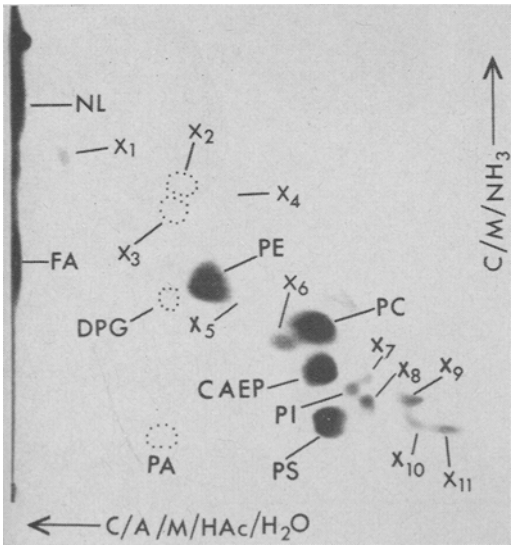

FIG. 1. Two-dimensional TLC of lipids of the sea anemone. Chloroform/methanol/28% aqueous ammonia 65/35/5 was used for development in the vertical direction followed by air-drying for 10 min and development in the horizontal direction with chloroform/acetone/methanol/acetic acid/water 5/2/1/1/0.5. Spots were localized by spraying with 55% by weight sulfuric acid containing 0.6% potassium dichromate and heating at 180C for 30 min. The sample (600 μg) was applied as a row of small spots over an area about 1 cm long in the lower right corner (placed 2.5 cm from each edge of the plate). Circled areas mark spots visible on the original chromatogram but not seen on the photograph.

RESULTS

Total Lipid and Phospholipid Distribution

The original lipid extract used in previous studies (6) and stored at −20C in chloroform/methanol 9/1 (50 mg/ml) was passed through a Sephadex column (10) to free it of nonlipid. A small amount (2.9%) of nonlipid was removed thus giving the value of 1.51% for the total lipid of the anemone rather than 1.56% previously reported (6).

The separation of lipid classes obtained by TLC is shown in Fig. 1 and the quantitative phospholipid distribution obtained by phosphorus analysis of the spots shown in Fig. 1 is presented in Table I.

Results of Prolonged Acid Hydrolysis

After acid hydrolysis under conditions for cleavage of phosphate esters leaving phosphonic acids intact, only CAEP was found to release a phosphonic acid. Phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine gave only choline, ethanolamine, and serine under these conditions thus demonstrating that these lipids are not phosphonolipids.

TABLE I

Phospholipid Composition of the Sea Anemone [a]

Lipid class	Percent of the total phospholipid
Phosphatidyl choline	22.0
Phosphatidyl ethanolamine	20.0
Phosphatidyl serine	13.6
Phosphatidyl inositol	2.5
Diphosphatidyl glycerol	1.0
Phosphatidic acid	0.3
Ceramide aminoethylphosphonate	19.9
X5	1.0
X6	0.5
X7	2.9
X8	2.7
X9	5.2
X10	3.1
X11	4.8

[a] The uncharacterized substances were designated X with numbers referring to spots marked in Fig. 1. The lipids X_1, X_2, X_3, and X_4 of Fig. 1 did not contain phosphorus.

Demonstration of the Presence of Sphingosine in CAEP

After acid hydrolysis of CAEP by the method of Gaver and Sweeley (14), TLC showed the presence of fatty acid methyl esters, unhydrolyzed CAEP, and a long chain base with the same migration as the authentic sample of sphingosine spotted on the same plate. No dihydrosphingosine was detected. Upon gas chromatography of the trimethylsilyl ether of the long chain base a retention time of 3.61 relative to methyl stearate was obtained. This is in good agreement with the retention time of 3.68 reported by Gaver and Sweeley (14) and confirmed by Feldman et al. (17).

Demonstration of Myristic Acid and Serine after Degradation of CAEP

The oxidative degradation procedure outlined in Diagram 1 yields myristic acid from C_5-C_{18} and serine from C_1-C_3 if the base is sphingosine and the aminoethylphosphonate moiety is linked to C_1 of sphingosine. Myristic acid was detected and identified as its methyl ester by gas chromatography on both polar and nonpolar columns. No other fatty acid was detected.

Serine was identified by its behavior on a Dowex 50 column and by TLC with three different solvents. The nitrogenous fraction before ion exchange resin column chromatography contained serine and four minor ninhydrin positive products. These minor components were readily separated from serine by ion

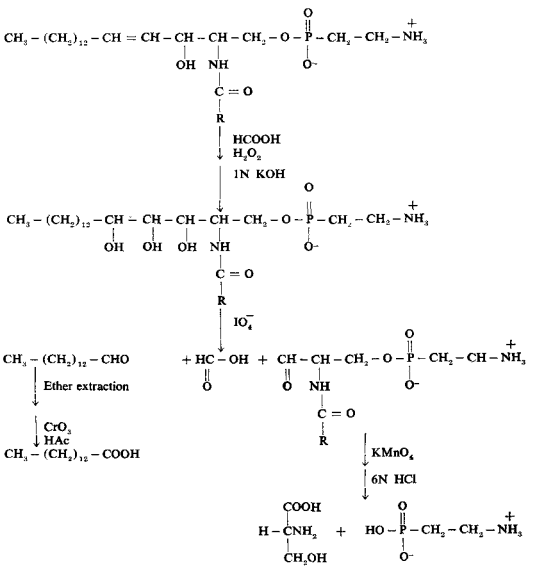

DIAGRAM 1.

exchange column chromatography. They were positive to the 55% sulfuric acid-0.6% potassium dichromate char spray (12) after TLC and thus presumably were unhydrolyzed lipid oxidation products. Evidence for the production of formic acid from C_4 was obtained by distillation after periodate oxidation when a positive Tollens test was obtained. Had the aminoethylphosphonate moiety been attached at C_3, β-aminodihydroxy butyric acid and/or β-hydroxy aspartic acid, both of which are easily distinguished from serine by column chromatography and TLC, would have been produced.

Fatty Acids of CAEP

Hydrolysis of CAEP with the methanol/water/hydrochloric acid mixture of Gaver and Sweeley (14) and with aqueous 2 N hydrochloric acid yielded the same fatty acids. The tentative identifications and amounts of the methyl esters are shown in Table II. Most fatty acids were saturated since the pattern was almost the same after hydrogenation over a palladium catalyst. The longer chain acids, particularly C_{24}, found in most animal sphingolipids appear to be absent. The small amount of unsaturated fatty acid is in keeping with the great stability observed for a preparation of CAEP that remained free of oxidation products for several years when stored in chloroform/methanol solution at −20C in a glass stoppered centrifuge tube.

TABLE II
Fatty Acid Composition of CAEP [a]

Fatty acid	Percent of total
14:0	0.5
15:br	1.3
15:0	0.4
16:0	82.4
16:1	2.3
17:br	7.3
18:0	0.6
18:2	3.8
18:3	0.5
?	0.9

[a] Carbon chain lengths are specified first and followed by double bond designations. Br indicates branched chain (tentative identification).

DISCUSSION

It is of interest that the sea anemone contains for the most part lipids typical of higher animals. CAEP is exceptional and may perhaps be viewed as a substitute for sphingomyelin present in most animal cells. Sphingomyelin is entirely absent from the anemone. It is also of interest that the molar ratio phosphatidyl choline/phosphatidyl ethanolamine/CAEP is very nearly 1/1/1. This ratio was observed to undergo marked alteration as a result of autolytic change. In our studies a frozen cake of sea anemone after storing at +4C for several days was found to contain about 20% free fatty acid with hydrolysis of most of the glycerolphospholipid. Only CAEP remained largely intact.

The absence of the C-P bond from the gly-

cerolphospholipids suggests that the biosynthetic routes for phosphatidyl ethanolamine and CAEP are different. It is possible that phosphatidyl ethanolamine arises solely by decarboxylation of phosphatidyl serine while CAEP is formed through the cytidine diphosphate pathway.

Establishment of the point of attachment of the polar group on CAEP as carbon one is of some importance with regard to cell membrane structure since the usual model formulations of membranes (18) assume attachment at carbon one. It is to be noted that polar groups on all sphingolipids are usually assumed to be linked through the primary hydroxyl group of carbon one of sphingosine and that the actual point of attachment appears only to have been demonstrated previously for brain sphingomyelin (16,22) and cerebroside (19,21).

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Role of ATP in the Inhibition of Lipogenesis in Fasted Animals

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ABSTRACT

This study was undertaken to obtain information both in vivo and in vitro on the role of adenosine triphosphate (ATP) in the inhibition of fatty acid synthesis in fasted mice.

Fasted mice were injected intravenously with glucose or fructose or intraperitoneally with insulin to increase the supply of endogenous ATP. They then received acetate 1-^{14}C, glucose 6-^{3}H or fructose U-^{14}C intravenously and were killed at various intervals. The controls received the labeled tracers only. The radioactivities of liver and carcass fatty acids were determined.

The action of ATP on homogenized livers from fasted mice was also determined.

The stimulation of lipogenesis was obtained under all these conditions, but only in animals fasted for 4 hr. Insulin was active only on the extrahepatic tissues. Fructose, as well as glucose, restored hepatic lipogenesis. In vitro, ATP restored lipogenesis by homogenized livers of mice fasted for 4 hr, but it inhibited the fatty acid synthesis by homogenized livers from unfasted mice.

The significance of the results is discussed.

INTRODUCTION

A GREAT NUMBER of factors have been suggested to be responsible for the inhibition of the synthesis of fatty acids during fasting. Among them are the decrease of activators, such as lipogenin (1) and citrate (2) or the accumulation of inhibitory substances such as fatty acids (3–6), a deficiency of necessary cofactors (ATP [7] or NADPH [8]) or even enzymatic damage (9–11). None of those factors can alone explain the decrease of lipogenesis.

A great deal of work concerning this subject has been carried out by means of in vitro experiments and with animals which have been fasted for long periods. We hoped to gain information on the original defect in lipogenesis by studying short-duration fasting. In our work the cofactor which received most attention was ATP. A deficiency of ATP as a reason for the inhibition of lipogenesis has been questioned (12).

The oxidation of fatty acids indeed yields the same amount of ATP as glucose itself, once they have been activated. Although the fasted animal derives its energy from the combustion of fatty acids, nevertheless its tissues contain much less ATP than a normally fed one (13). A possible explanation for the phenomenon is the accumulation of fatty acids during fasting which could, perhaps, uncouple oxidative phosphorylation (14–15) and reduce the supply of ATP.

Further, a decrease of utilization of the ATP produced by the catabolism of fatty acids could result from the existence of different ATP compartments (16–17), or from an increase in the activity of microsomal ATPase, as is proposed by Masoro (18). However, the addition of ATP to liver homogenates of rats fasted for 24 hr did not permit Masoro (19) to restore the synthesis from acetate 1-^{14}C.

To avoid the risk of hydrolysis and of pharmacological actions we did not inject ATP but indirectly increased the supply of ATP by loading the animal with glucose and fructose, and by giving insulin injections. We also carried out a direct assay in vitro by incubating ATP in the presence of homogenized livers of mice fasted for brief periods.

EXPERIMENTAL PROCEDURE

A first experiment was conducted on mice (female, 30 g) which had been fed ad libitum and fasted for either 4 or 24 hr. Some of them were injected intraperitoneally (i.p.) with 0.5 I.U. of Novo insulin (glucagon-free). One hour later, they received intravenously 3.3 μc of acetate 1-^{14}C (29 mc/mmole) along with 40 μc of glucose 6-^{3}H (1.22 curies/mmole) and were killed by decapitation 3 min after the injection. The livers were excised quickly and treated immediately for the isolation of glycogen. Glycogen was extracted

TABLE I

Reestablishment by Insulin of Inhibited Fatty Acid Synthesis (4 Hour-Fasted Animals)

	Carcass		Liver		Liver		
	Total radioactivity of fatty acids		Total radioactivity of fatty acids		Glycogen (%)	Total radioactivity of glycogen ^3H	Fatty acids (%)
	^{14}C	^3H	^{14}C	^3H			
Controls	588	132	48.6	6.2	0.62	2.94	3.76
	↑ $p<0.001$	↑ $p<0.001$	↑ $p<0.001$	↑ $p<0.001$	↑ n.s.	↑ n.s.	↑ $p<0.05$
4 hr–fast	↓ 269	↓ 50.5	↓ 13.4	↓ 1.9	↓ 0.49	↓ 1.62	↓ 4.4
	↑ $p<0.001$	↑ $p<0.001$	↑ n.s.	↑ n.s.	↑ $p<0.01$	↑ $p<0.05$	↑ n.s.
4 hr–fast + insulin	↓ 596	↓ 316	↓ 16.0	↓ 3.3	↓ 0.19	↓ 0.78	↓ 4.34

Mice (female, 30 g) were fed a fat-free diet for 8 days. They received intravenously 3.3 μc of acetate 1-^{14}C and 40 μc of glucose 6 ^3H and were sacrificed 3 min after the injections. The results are expressed in cpm 10^{-3}. Insulin was administered intraperitoneally 1 hr before the labeled precursors.

in the usual way, precipitated from the KOH solution by alcohol, and transformed into acetyl-glycogen (20) for the determination of radioactivity. The acetyl-glycogen was dissolved in acetic acid and counted in a Packard Tri-Carb liquid scintillation spectrometer (5 g of PPO and 300 mg of POPOP per 1 of toluene). Fatty acids of the liver and carcass were extracted with petroleum ether after removal of nonsaponifiable lipids. The extracts were dried by evaporation under nitrogen and counted using the same scintillation mixture. "Quenching" was estimated by the discriminator ratio-method of Hendler (21).

In a second experiment, mice (female, 30 g) fasted for 4 hr received simultaneously an injection of 30 mg of unlabeled fructose or glucose and 10 μc of labeled fructose U–^{14}C (3 mc/mmole) and were killed either 6, 10, or 30 min after the administration. The fatty acids were extracted and counted for radioactivity as described above.

In the in vitro experiment, the livers were homogenized with 2 volumes of sucrose solution (0.25 M) in a teflon homogenizer of the "Potter" type. Three milliliters of homogenate were used for each sample and incubated at 37C for 1 hour with 2 ml of a medium described by Masoro (19) in which potassium succinate was replaced by sodium succinate and isocitrate by citrate. In contrast with Masoro's technique, the whole homogenate was used, i.e. without eliminating the nuclei and cell fragments. Its pH was adjusted to 7. Five microcuries of acetate 1–^{14}C were added to each sample, some with and some without ATP (23.8 mg or 47.6 mg). The incubation was stopped by hydrolysis with alcoholic KOH. After removal of nonsaponifiable material with petroleum ether the mixture was acidified and the fatty acids were extracted with petroleum ether.

The other experimental conditions are given in the legends of the tables.

RESULTS

Effect of Insulin on Fatty Acid Synthesis; Synthesis of Hepatic Glycogen

In the carcass, the i.p. administration of 0.5 I.U. of insulin into mice fasted for 4 hr. completely reversed the inhibition of fatty acid synthesis (seen in the fasted controls) from acetate 1–^{14}C and glucose 6–^3H; it provoked a synthesis from glucose 6–^3H even greater than in the unfasted mice (Table I).

In contrast, this hormone produced no significant change in hepatic lipogenesis. If the mice had been deprived of food for 24 hr, insulin did not even restore lipogenesis in the carcass.

On the other hand, the amount of hepatic glycogen per unit weight and its synthesis from glucose decreased in animals which had received i.p. injections of insulin. Similar results were obtained by Rafaelsen et al. (22) who also found that insulin increased the synthesis of fatty acids in adipose tissue of fasted rats.

Effect of Glucose or Fructose on Fatty Acid Synthesis After a Short Fast

The incorporation of fructose U–^{14}C into the fatty acids of liver and carcass was studied in mice fasted for 4 hr which received simultaneously an unlabeled load of glucose or fructose and killed 6, 12 and 30 min after this

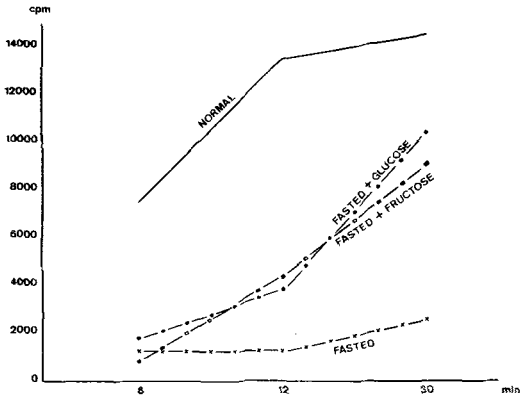

FIG. 1. Four-hour fasted mice received simultaneously an injection of 30 mg of unlabeled fructose or glucose and 10 μc of labeled fructose U-^{14}C and were killed 6 min, 10 min and 30 min after the administration.

injection (Fig. 1). When fatty acid synthesis was measured 30 min after fructose injection, hepatic lipogenesis from this precursor was strongly reduced in fasted animals (83% inhibition in the liver and 60% in the carcass). The addition of unlabeled glucose or fructose caused an increase in lipogenesis in the livers of fasted mice. This increase was not immediate but progressive and already significant 12 min after the injection ($p<0.001$). The load of fructose seemed to dilute the radioactivity in hepatic fatty acids, if mice were killed 6 min after the injection, but this decrease was not significant. In the carcass, only glucose favored the lipogenesis and only in the mice killed 30 min after the administration of the labeled precursor.

Effect of ATP on Fatty Acid Synthesis in Presence of Mouse Liver Homogenates

In Table II it is shown that the fasted animals (4 hr) the ATP significantly increased the synthesis (mean : 42.2% for an amount of 23.8 mg of ATP per sample, and 208% for twice that amount of ATP).

An excess of ATP significantly decreased (about 66%) the lipogenesis in liver homogenates of fed animals in our experimental conditions (pH 7).

DISCUSSION

If a deficiency in ATP is responsible for the inhibition on lipogenesis, we would have a good chance to unmask it by studying the effects of short-fasting times. Indeed, the mobilization of fatty acids, if measurable in some degree by the increase of hepatic fatty acids, is subsequent to the inhibition of the lipogenesis. Thus one can assume that when exogenous carbohydrate is not available, or once the reserves of glycogen have been spent, a deficiency in ATP and in lipogenesis can occur. Fatty acid synthesis decreases 75% in the liver and 58% in the carcass of animals fasted for 4 hr if they are fed with a fat-free diet beforehand (23).

The administration of insulin was intended to favor the intracellular penetration of glucose and to produce an enrichment in ATP. A total restoration of fatty acid synthesis was noted

TABLE II

Effect of ATP on the Synthesis of Fatty Acids in the Presence of Mouse-liver Homogenates

	Total radioactivity of fatty acids in cpm.				
	Fast	Fast + ATP	(23.8 mg per sample)	Fast + ATP	(47.6 mg per sample)
I	37.800	61.000	(+ 61.5%)	126.000	(+ 233%)
	39.200	58.000	(+ 48.0%)	111.200	(+ 183%)
II	31.100	41.300	(+ 32.8%)		
	34.100	48.000	(+ 40.7%)		
	36.300	46.500	(+ 28.2%)		
Average			(+ 42.2%)		(+ 208%)
	Normal	Normal + ATP (23.8 mg)			
	91.200	38.000	(—57 %)		
	77.600	29.700	(—61.8%)		
	79.000	25.500	(—67.8%)		
	183.000	41.500	(—77.3%)		
Average			(—65.97%)		

Mice received 1.5 g dry bread at 6:00 PM and 100 mg at 5:00 AM the next day. Fed mice received a supplementary meal (100 mg bread) at 8:15 AM. All animals were killed at 9:00 AM. Other conditions: see experimental procedure.

in the carcass only but not in the liver of mice which had been fasted for 4 hr.

An intravenous load of glucose or fructose after a brief fast enhances lipogenesis in the liver. The similar results with these two sugars excludes an indirect involvement of insulin, since fructose would not be able to favor the secretion of insulin to the same extent as glucose. At least the response to the load should not mainly affect the liver.

These results suggest that the load of glucose and fructose may have increased the ATP to the necessary level in our animals fasted for a short time. In our experimental conditions, only the glucose restored the synthesis in the carcass.

The difference may be explained by a slower rate of metabolism of fructose in the extrahepatic tissues (24). However it should be pointed out that injected fructose does not dilute fatty acid synthesis as it does in the fed animals (25). This absence of dilution in fasted animals could have the same meaning as a stimulation of synthesis.

The results of our in vitro experiment strongly suggest that ATP is a limiting factor in fasting, although Masoro (19) was unable to restore lipogenesis in his comparable experiments. His animals were fasted for a much longer time than ours and enzymatic changes were possibly more extreme. In fact, even in vivo we could not immediately reestablish lipogenesis after a prolonged fasting of 24 hr.

Another substance, bicarbonate, is able to restore the synthesis of fatty acids in brieflyfasted animals (26) and although its action as a substrate has to be considered, it seems more probable that bicarbonate interferes again in relation with ATP, maybe as an activator, confirming the theory proposed by Kaziro (27). It could also favor all reactions catalyzed by ATP phosphotransferases, especially those catalyzed by hexokinase. According to other experiments (results to be published) it also seems that the glycolytic enzymes must be present in sufficient quantities for HCO_3 to enhance synthesis of fatty acids.

Our present experiments do not enable us to explain the inhibition of fatty acid synthesis by ATP in normally fed mice, but there is little doubt about its enhancing role in fasted animals and its decrease during fasting could be the factor, or one of the primary factors, directly or indirectly responsible for the inhibition of the synthesis. This conclusion would also be in agreement with a recent suggestion of Wakil (28): the formation of phosphorylated sugars may activate the indispensable enzymes for the synthesis of fatty acids.

ACKNOWLEDGMENT

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Studies on the Lipids of Sheep Red Blood Cells. I. Lipid Composition in Low and High Potassium Red Cells

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ABSTRACT

The lipid composition of whole red blood cells was investigated in five sheep with red cells containing a low concentration of potassium (LK) and in five sheep with red cells containing a high concentration of potassium (HK). No apparent differences within the limit of error of the experiment were detected in the lipid class composition between the HK and LK red cells. Cholesterol, the only nonpolar lipid detected in the tissue, was present in one-to-one molar ratio to the total phospholipids. Phosphatidyl ethanolamine and sphingomyelin accounted for 85% of the total phospholipids; phosphatidyl serine, phosphatidyl inositol, phosphatidic acid, and lysolecithin were present in lesser amounts. No lecithin was detected in any of the animals in this investigation. Plasmalogen compounds were found only in the ethanolamine lipids. The molar ratio of choline to noncholine phospholipids was also approximately one to one. It was concluded that the major lipid class distribution in the two types of red cells cannot be directly responsible for the differences observed in the cation concentrations in these cells in the two species of sheep.

INTRODUCTION

THE LIPID COMPOSITION of the red cell has been extensively investigated (1), initially because of the relative accessibility of the tissue, and more recently because of increasing interest in the erythrocyte stroma as a model membrane system. Human red cells have been studied most frequently, but increasing interest in the erythrocytes of other species has resulted in a considerable body of data (2-4). Sheep have been studied by several workers in the past decade(2, 5-7). Turner (8,9) reported that lecithin was absent in the red cells of oxen, sheep, and goats, and speculated that this accounted for the resistance to snake venom lysis reported by previous workers (10), although this has now been disputed (11).

The sheep erythrocyte exhibits an interesting anomaly in its ability to transport sodium actively (12), giving rise to two types of cells in sheep, the high potassium (HK) and low potassium (LK) varieties. Previous reports on the lipid composition of sheep red cells have not compared the lipids in relation to differences in the sodium transport system, except that two brief statements that no differences were found (13,14) have appeared in reports concerned primarily with other matters. In neither case were any quantitative data presented. In this work, a comparative study of the detailed lipid composition of erythrocytes in the two types of sheep is presented.

EXPERIMENTAL

Sampling and Isolation of Red Cells

A flock of purebred Hampshire sheep was maintained at the laboratory under standard sheep-raising conditions except that the animals were constrained to a small plot and fed on alfalfa with a grain supplement. They were classified as LK or HK on the basis of serum and red-cell sodium and potassium levels as determined by a flame photometric technique (15), and each animal was tagged for identification.

Whole fresh blood was drawn by venipuncture into sterile plastic blood bags (Fenwal Laboratories, Morton Grove, Ill.) to which was added previously 0.5 ml of heparin (ammonium salt) solution (1000 USP units/ml, American Hospital Supply Corporation, Evanston, Ill.) for each 100 ml of blood being drawn. Little or no hemolysis or clotting was observed with this technique, although severe hemolysis was observed in one case in which a plasticizer (dioctyl phthalate) was recovered in the lipid extract; it may have come from the plastic blood bag. If the blood could not be processed within 3 hr after drawing, it was discarded. In most cases 150 to 200 ml of blood was taken from an adult animal.

The blood was transferred to 50-ml polypropylene centrifuge tubes and cooled to 0C in an ice bath. The cells were separated from the serum by centrifugation at 0C for 15 min at 2500 rpm in an International Size 2 centrifuge fitted with a 16-place rotor. The serum was removed by aspiration and the cells were resuspended in 310 milliosmolar phosphate buffer, pH 7.4, and recentrifuged three times. After each centrifugation the buffy layer and top 2-3 mm of cells were discarded to insure complete removal of leukocytes and platelets. The packed cells were then transferred to lyophilization

flasks, frozen in a dry-ice and acetone bath and lyophilized to remove all water from the cells.

Extraction of Lipids

A weighed portion of lyophilized red cells, usually 10 g, was ground briefly in a mortar and added slowly to a 2000-ml volumetric flask containing 1200 ml of methanol-chloroform (1/1, v/v), which was being stirred vigorously by a magnetic mixer. After addition of the red cells, chloroform was added to give a final extraction solution of chloroform-methanol approximately 2/1, v/v. Stirring was continued for 5 min; then the solution was filtered through a fast filter paper (Sharkskin Grade, 18.5-cm circles, Carl Schleicher and Schuell Company, New York, N. Y.) which had been previously washed with methanol and chloroform to remove an organic residue often found in filter paper (16). The volume of solvent recovered after filtration was recorded for calculation of the total lipid in the sample (17), and the solution was transferred to a separatory funnel. Distilled water (20% by volume) was added, the separatory funnel was shaken vigorously for several minutes, and the phases were allowed to separate overnight in a cold room at 4C. The organic phase was then transferred to a rotary evaporator and the solvent was removed at reduced pressure with the temperature below 40C. Before the sample became completely dry it was transferred to a tared vial and the remaining solvent was removed under nitrogen. The sample was desiccated in vacuum overnight and then weighed.

Thin-layer Chromatography

Both the total lipid extract and the fractions obtained from column chromatography (see below) were subjected to one- and two-dimensional thin-layer chromatography (TLC) on Silica Gel H or HR, obtained from Brinkmann Instruments, Westbury, New York. In some cases 10% $MgSiO_2$ was added to the absorbent, and then the sample was mixed in a ball mill for 15 min to insure even distribution of the components.

Neutral lipids were chromatographed one-dimensionally using hexane-diethyl ether-acetic acid (85/15/2, v/v/v) (18). Polar lipids were chromatographed one-dimensionally using chloroform-methanol-water (65/25/4, v/v/v).

Two-dimensional chromatography was done using the methods of Rouser et al. (19) involving two pairs of solvent systems. The first set used chloroform-methanol-aqueous ammonia (65/35/5, v/v/v), followed by chloroform-acetone-methanol-acetic acid-water (5/2/1/1/0.5, v/v/v/v/v); the second set used chloroform-methanol-water (65/25/4, v/v/v), followed by *n*-butanol-acetic acid-water (60/20/20, v/v/v).

Spots were visualized by exposure to iodine vapor or by spraying with 0.6% potassium chromate in 50% sulfuric acid. Lipids containing amino groups were detected with ninhydrin spray. Phospholipids were detected with a spray reagent containing phosphomolybdic acid for the visualization of phosphorus compounds. Both spray reagents were obtained from Brinkmann Instruments, Westbury, New York. Choline-containing compounds were detected with Dragendorff's bismuth-iodine stain (20).

Permanent records of the thin-layer plates were made by photographing the spots with a 4 × 5 Crown Graphic Camera equipped with a Polaroid Corporation 4 × 5 film pack adapter, using a Polaroid 3000-speed, type-57 film with back illumination from a transparency illuminator.

Column Chromatography

A portion of the total lipid extract was separated by column chromatography on 6.0 mm × 900 mm silica gel columns. The packing was prepared as described previously (21). The details of column construction and column preparation are described elsewhere (22).

The lipids were separated using an automatic system (22) that produced concave gradients of methanol in chloroform or hexane. The cholesterol and neutral lipids were removed from the column by elution with chloroform prior to gradient elution. The load factor was approximately 7 mg of total lipid extract per gram of silica gel. The charge was added to the column in a minimal volume of chloroform; 1000 ml of chloroform was found to remove all the neutral lipids from the column without appreciably affecting the migration of polar constituents (23). A concave gradient of methanol in chloroform was found to yield the best separations with this extract.

Ten milliliters were collected in each collection tube during the gradient elution chromatography. Appropriate portions were removed from each tube for phosphorus determination. One-dimensional TLC was also used to monitor the column. The peaks obtained from the column were identified by infrared spectrophotometry coupled with two-dimensional TLC.

When the major elution peaks had been located by the above procedures, the corresponding fractions were isolated by pooling the appropriate collection tubes. The solvents were

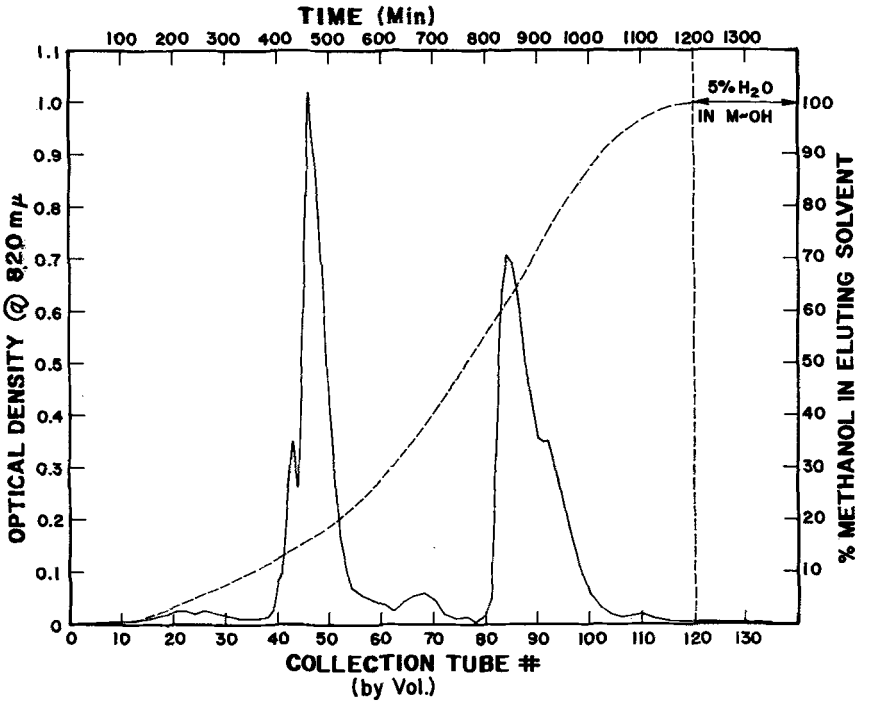

FIG. 1. Typical gradient elution column chromatography of sheep red cell phospholipids. Solid line: optical density (left ordinate) resulting from phosphorus analysis of 0.2 ml of solution from individual collection tubes. Dashed line: composition of elution solvent (right ordinate) indicating the shape of the gradient.

then removed in a rotary evaporator as described in the extraction procedure. The residues were transferred to tared vials through a sintered glass funnel to remove any silica. Triplicate phosphorus determinations were then performed on each fraction, and two-dimensional TLC as well as infrared spectrophotometry were done to characterize the fractions further. Fig. 1 presents a typical run in this system.

Infrared Spectrophotometry

All spectra were obtained with a Perkin-Elmer Model-521 grating infrared spectrophotometer. Some samples were dissolved in organic solvent and read in sealed solvent cells having sodium chloride windows, with 1.0- or 0.1-mm path lengths depending on the amounts of material available. Others were mixed with potassium bromide and pressed into disks (5 mm diameter) of 0.25 to 0.5% sample in 50 mg KBr. The disks were used in conjunction mg a 4× potassium bromide refracting beam condenser (Perkin-Elmer Corporation, Norwalk, Conn.) in the sample beam of the instrument. In some instances a film of the sample was formed on NaCl plates by evaporation from a suitable solvent.

When the spectrum was obtained on the sample in solution it was possible to obtain quantitative data by comparing the specific absorbances at certain wavelengths with that of a standard compound obtained from a commercial source or purified material prepared from natural sources. Spectra obtained from the pressed disk or thin film technique were used only for qualitative identifications. Commercial standards (prepared from various organs) for phosphatidic acid, phosphatidyl serine, phosphatidyl ethanolamine, lecithin, and lysolecithin were obtained from General Biochemicals, Chagrin Falls, Ohio, sphingomyelin from Arnel Products, Inc., New York, N. Y., and cholesterol from Applied Science Laboratories, State College, Pa. Myophosphatidyl inositol was a gift from C. Ballou.

Chemical Analysis

In addition to the infrared and chromatographic analyses described above, specific chemical tests were performed for phosphorus and cholesterol. Phosphorus was analyzed by a modification of Bartlett's procedure (24) using a mixture of perchloric and sulfuric acids

for the digestion step. Cholesterol was determined by the method of Hanel and Dam (25).

RESULTS

In initial experiments the washed red cells were first hemolyzed as described by Dodge et al. (26). This had the advantage of removing most of the heme pigments that are carried into the lipid extract in the direct extraction procedure. However, there was the possibility of loss of some lipid during the hemolysis (2), and the hemolysis procedure added considerable time to the overall extraction. Direct extraction therefore seemed preferable and was adopted as the standard procedure.

While the lyophilization step increased the time of the procedure, it had three distinct advantages. Considerably less heme pigment and other nonlipid material was extracted from the dried red cells than from fresh preparations, less lipid remained in the aqueous phase during the washing step, and, finally, the emulsion resulting from the aqueous wash of organic extract was much less stable than when wet red cells were used. The emulsion resulting from dried material usually broke completely in 4 hr, and at the most cleared on standing overnight, while that from wet cells was stable for 2 to 3 days. Thus the additional time needed for the lyophilization step was usually regained during the separation of the organic and aqueous phases.

Table I presents recovery data for the extraction procedure. The data are from an experiment in which the upper aqueous phase as well as the organic solvent phase from the extraction was evaporated to dryness; the material recovered was transferred to a tared vial and weighed as described for the organic phase. In addition the insoluble proteinaceous material retained in the filter paper was reextracted as described for the dried cells and then subjected to digestion with 2 N KOH at 60C for 7 days. The digestion mixture was then neutralized with HCl and the aqueous phase was extracted with hexane followed by chloroform.

TABLE I
Extraction and Recovery Data from Sheep Lyophilized Red Blood Cells

Volume of packed red cells	23.4	ml
Weight of dried cells	10.44	g
Weight of total lipid extract	164.0	mg
Weight of residue in aqueous phase	70.3	mg
Organic-solvent-soluble material in aqueous phase	0.1	mg
Hexane-soluble material after KOH digestion of extracted cells	0.17	mg
Chloroform-soluble materials after KOH digestion of extracted cells	1.30	mg

The material found in the aqueous phase of the initial extraction was primarily free amino acids and low molecular weight polypeptides (di, tri, tetra). Free phosphoryl ethanolamine and phosphoryl serine were also detected along with inorganic material.

The lipid present in the proteinaceous residue correspond in amount to that expected from solvent hold-up and did not differ in composition from the lipid in the organic phase. Hence, it is believed that the extraction procedure recovered essentially all of the lipid from the cells and that little nonlipid material was carried into the lipid extract.

Table II gives the total lipid, cholesterol, and phospholipid of the red cells from five HK and five LK sheep. Cholesterol was the only neutral

TABLE II
The Potassium and Sodium Ion Concentration, Total Lipid, Cholesterol, and Phospholipid Content of Sheep Red Cells in HK and LK Sheep

LK Sheep No.	Sex	Meq. Na^+ per ml packed RBC	Meq. K^+ per ml packed RBC	Total lipid (mg/g dry RBC mass)	Cholesterol (mg/g dry RBC mass)	Phospholipid (mg/g dry RBC mass)
1	M	102	1	15.79	5.17	10.62
2	M	115	8	15.61	5.16	10.45
3	M	97	1	14.20	4.60	9.80
5	F	114	12	14.68	4.83	9.88
9	F	120	10	15.60	4.85	10.75
Average		110	6	15.18	4.92	10.30
HK Sheep No.						
4	F	28	60	15.32	5.05	10.22
6	F	29	60	14.68	4.78	10.00
7	F	23	54	16.28	5.34	10.96
8	M	41	61	15.62	5.27	10.29
10	F	29	59	15.16	4.98	10.18
Average		30	59	15.34	5.08	10.33

TABLE III
Chromatographic Fractions Obtained by Combining Individual Collection Tubes from the Gradient Elution Column Chromatography

Fraction No.	Tube No.	Total weight (mg)	Phosphorus (%)	Tentative identification
1	1–39	8.6	1.5	Phosphatidic acid
2	40–43	6.9	3.2	Phosphatidyl serine
3	44–61	37.0	4.1	Phosphatidyl ethanolamine[a] and phosphatidyl serine
4	62–79	5.4	2.4	Phosphatidyl inositol and phosphatidyl ethanolamine
5	80–105	51.9	3.9	Sphingomyelin
6	106–115	1.0	3.3	Sphingomyelin + lysolecithin
7	116–135	0.5	0.0	?

[a] Contained ethanolamine plasmalogen.

lipid detected in the red cells of these sheep; no cholesterol esters, triglycerides or free fatty acids were present. Traces of hydrocarbon were found in all cases, but it seems reasonable to assume it to be an artifact of the extraction procedure. The cholesterol fraction (melting point 145C ± 2C, obtained by using a Buchi melting point apparatus with melting point capillary open to the atmosphere) was also analyzed by gas chromatography on an SE-30 column at 275C; no significant peaks other than that corresponding to cholesterol were detected even when the column was overloaded by a factor of 20.

The phospholipid composition was determined by a combination of TLC and column chromatography combined with infrared spectrophotometry. The gradient elution pattern (Fig. 1) was the primary basis of the analysis. Individual collection tubes were pooled to form the seven fractions listed in Table III. The percentage of phospholipid was determined by phosphorus analysis of the lipid residues after the fraction had been identified by TLC and infrared spectrophotometry. Fig. 2A shows a two-dimensional TLC of the total lipid extract of the sheep red cell. Fraction 1 contained only a single spot by two-dimensional TLC and moved with standard reference phosphatidic acid. In the column chromatography a consid-

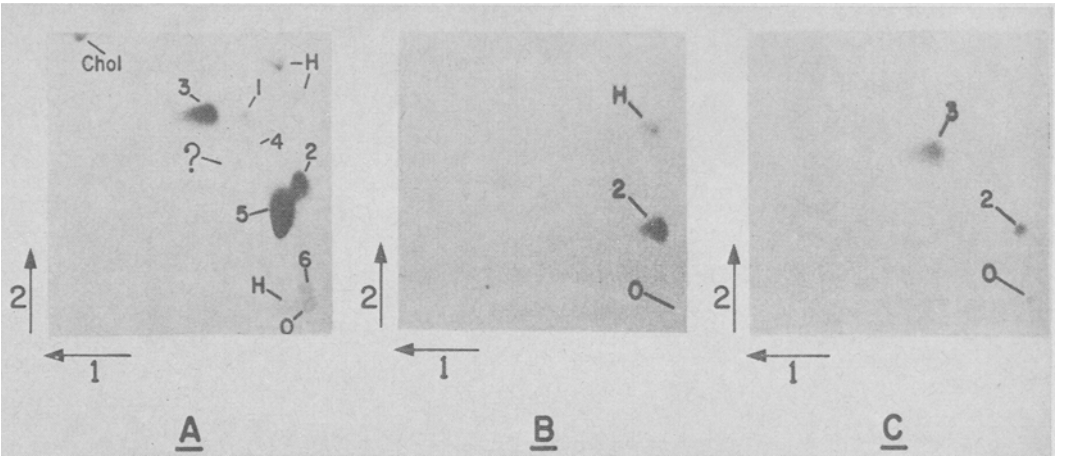

FIG. 2, A-C: Spot No. and tentative identifications: O, origin, heme pigments; 1, phosphatidic acid; 2, phosphatidyl serine; 3, phosphatidyl ethanolamine; 4, phosphatidyl inositol; 5, sphingomyelin; 6, lysolecithin; ?, unknown, no phosphorous; H, heme pigments; Chol, cholesterol.

FIG. *2A*. Two-dimensional TLC of total lipid extract of sheep red blood cells; 744 μg was applied at the origin. The developing solvents were: (1) chloroform-methanol-ammonium, 65/35/5, and (2) chloroform-acetone-methanol-acetic acid-water, 5/2/1/1/0.5. The spots were developed with iodine vapor. *2B*. Two-dimensional TLC of fraction 2 (pooled tubes 40-43 from gradient elution chromatography, Fig. 1), developed with the same solvents as in Fig 2A and stained with ninhydrin spray reagent. *2C*. Two-dimensional TLC of fraction 3 (pooled tubes 40-43 from gradient elution chromatography, Fig. 1), developed as in Fig. 2A and stained with iodine vapor.

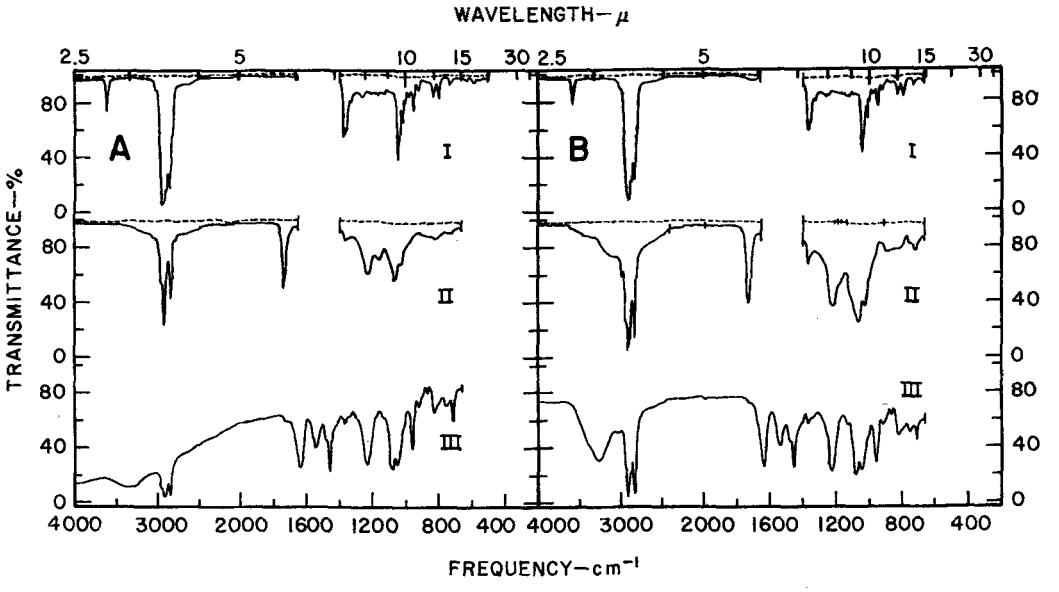

FIG. 3A. Infrared spectra of reference compounds obtained from commercial sources. I, cholesterol in carbon disulfide solution; II, phosphatidyl ethanolamine in carbon disulfide solution; III, sphingomyelin in KBr disk. Carbon disulfide obscures the region of the spectrum between 1660 and 1400 cm^{-1}. *B*. Infrared spectra of lipids from sheep red cells isolated by gradient elution column chromatography. I, cholesterol in carbon disulfide solution; II, phosphatidyl ethanolamine and ethanolamine plasmalogen mixture in carbon disulfide solution; III, sphingomyelin, solid film on NaCl plate. Note that curve II differs from the curve for the pure compound (II in Fig. 3A) in the ratio of the transmittance of ester absorption at 1740 cm^{-1} to that of phosphate absorption at 1100 cm^{-1}.

erable amount of heme pigment was eluted with this fraction; infrared and phosphorus values were consistent, although weight values were 50 to 100% higher. Fraction 2 contained only phosphatidyl serine; no plasmalogen or phosphatidyl ethanolamine were detected. Fraction 3 was primarily phosphatidyl ethanolamine and ethanolamine plasmalogen with a trace of phosphatidyl serine (Fig. 2B and 2C). The percentage of serine in this fraction was estimated by scraping the spot from the thin-layer plate and determining the phosphorus content as described by Rouser et al. (19). Fraction 4 was primarily phosphatidyl inositol but also contained some phosphatidyl ethanolamine. Fraction 5 was sphingomyelin. Fraction 6 was a mixture of sphingomyelin and lysolecithin; the percentages of sphingomyelin and lysolecithin were estimated by the technique of Rouser et al. (19). Fraction 7 contained no phosphorus. Infrared spectra of reference compounds are compared with the major lipids isolated from the red cells in Fig. 3A and 3B, respectively.

TABLE IV
Average Phospholipid Composition of HK and LK Sheep Red Blood Cells[a]

Phospholipid	% Total phospholipids	
	HK	LK
Phosphatidic acid	3.4 (2.6–3.9)	2.8 (2.4–3.8)
Phosphatidyl serine	8.5 (7.7–10.0)	7.9 (7.0–9.1)
Phosphatidyl ethanolamine[b]	32.2 (27.4–33.8)	33.2 (26.8–34.5)
Phosphatidyl inositol	3.0 (1.8–3.4)	3.4 (3.0–3.9)
Lecithin	0.0	0.0
Sphingomyelin	52.4 (46.8–55.1)	52.2 (45.9–54.7)
Lysolecithin	0.5 (0.3–0.7)	0.5 (0.4–0.6)

[a] Five animals per group, range of values given in parentheses following average value.
[b] Contains ethanolamine plasmalogen also.

TABLE V
Comparison of Literature Values for the Phospholipid Composition of Sheep Red Blood Cells

Phospholipid	% of Total phospholipids				
	References				
	(2)	(5,35)	(6)	(7)	This work
Phosphatidic acid	0.3				3.1
Phosphatidyl serine	12.9	40.8	38	36	8.2
Phosphatidyl ethanolamine[a]	5.6				32.7
Phosphatidyl inositol	3.8	0.7			3.2
Lecithin	4.4[b]	2.9	5	1	0.0
Sphingomyelin	48.0	56.0	50	63	52.3
Lysolecithin		0.6	7		0.5

[a] Includes ethanolamine plasmalogens.

[b] Includes lysolecithin.

The phospholipid class composition for the 10 animals is given in Table IV. Phosphatidyl ethanolamine and phosphatidyl serine along with sphingomyelin were the primary phospholipids, while phosphatidic acid, phosphatidyl inositol, and lysolecithin were present in smaller amounts. No lecithin was detected in any of these animals.

Plasmalogens were detected only in the phosphatidyl ethanolamine fraction. No attempt was made at present to quantitate the ratio of plasmalogen to diacyl compounds. The presence of lysolecithin without lecithin suggests that it may have been an artifact of the chromatographic or the extraction procedure. However, a spot of lysolecithin appeared on thin-layer chromatograms of the unfractionated total lipid extract for every animal in the series (Fig. 2A). It has been suggested (27) that heparin, used as an anticoagulant, may cause degradation of red cell lipids to lyso compounds. Recently Dodge and Phillips (28), refuting this assumption, asserted that lyso compounds are not produced as artifacts in red cell lipid extract but, rather, an autoxidation product is formed as a product of oxygen contact only. Hence the lysolecithin in sheep red cells would seem to be a real component of the cell.

No significant differences are apparent in the distributions of the major lipid classes between the two types of erythrocytes. Some small variations in acidic lipids can be observed in the average values for the two species (Table IV), but the ranges were practically identical. These differences would probably disappear with a larger series of animals and improved methods.

DISCUSSION

Two previous reports in the literature (13, 14), which stated that the lipid class composition did not differ in HK and LK sheep red cells, are confirmed in this work in which quantitative values are given for the animals under investigation. In addition, our values for the lipid composition of sheep red cells are in agreement with those reported by previous workers (5-9, 11). All of the previous reports indicated that in the sheep, as in other ruminants (3,7,9), the lecithin content is lower than in man or the carnivores (4,9,29). While no lecithin was detected in the animals studied here, various workers have reported values from 0 to 12% for the sheep erythrocyte (2,7). Table V compares the data from earlier work with that obtained here. Genetic differences among different flocks of sheep may be responsible for variation in the lecithin content of erythrocytes, particularly since sheep are a highly inbred species. This point may well deserve further study.

The molar ratios of sphingomyelin, acidic phospholipids and cholesterol in the cell are 1/1/2; hence, there is one cholesterol molecule for each phospholipid molecule. If the lipids in the red cell are indeed arranged in a bimolecular leaflet as first proposed by Görter and Grendel (30) and independently by Davson and Danielli (31), these molecular ratios are consistent with the theory of membrane structure proposed by Vandenheuvel (32), which postulates the existence of phospholipid-cholesterol complexes. In this theory, membrane ion transport phenomena can be accounted for by charge-ordering in the arrangement of the polar groups in the phospholipid and protein molecules. In view of the data obtaind here it seems unlikely that the distribution of the phospholipid classes in the sheep red cell is related directly to the difference in ability to transport sodium.

On the other hand, the observed similarities in gross cholesterol and phospholipid composition in the two species do not rule out the

involvement of lipids in the transport mechanism of the membrane. This and most other studies thus far give only a static picture of the membrane. It will be interesting to investigate phospholipid metabolism in these animals. Indeed, Jacobs and Karnovsky (33) recently reported that phospholipids of human red cells in hereditary spherocytosis have different metabolic rates (as measured by the incorporation of P^{32}) than those of normal cells, and implicated specifically the phosphatidyl serine fraction.

In addition, most previous reports on sheep red cells tended to group the lipid into three classes: cephalins, lecithins, and sphingomyelin. This grouping tends to obscure the complexity of the cephalin group, which is perhaps better termed acidic phospholipids. In sheep, for example, this group includes at least four members: phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol; plasmalogen analogues and possibly the glyceryl ether compounds are present also. The vast number of possible combinations and permutations could conceivably lead to subtle differences, hard to detect even with the most sophisticated techniques available today. This does not even include differences that might exist in the fatty acids associated with the various phospholipids, which may also be involved in transport; fatty acid composition is known to influence the permeability of the red cell (34).

Tosteson et al. (14), in their recent study of the soluble lipoprotein fragments of red cell membrane in HK and LK sheep, were unable to relate ATPase activity of the fragments to the sodium pump in the intact cell. They were forced to the conclusion that structural differences in the membrane as a whole were probably more important than specific enzyme concentrations in the cell. More and better analyses of the red cell components may eventually provide an answer to this problem.

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Technical assistance by Robert Booth; advice concerning the intricacies of two-dimensional TLC technique provided by George Rouser; sodium and potassium determinations performed by Miss Marie Laskaris.

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Separation of Cholesterol-Desmosterol Acetates by Thin-Layer and Column Chromatography on Silica Gel G-Silver Nitrate

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ABSTRACT

A chromatographic method, using columns packed with Celite-Silica Gel G impregnated with silver nitrate, has been developed for the separation of cholesterol from desmosterol (24-dehydrocholesterol).

The system employed allows a complete separation of the two sterols as acetates, as determined by gas-liquid chromatography and thin-layer chromatography.

The recovery of pure cholesterol and desmosterol is quantitative.

INTRODUCTION

THE RECENT DISCOVERY of drugs that induce an accumulation of sterol precursors of cholesterol (1-3), has focused attention on the problem of the separation and recovery of these sterols. The gas chromatographic method developed by Horning et al. (4) permits a complete separation of desmosterol ($\Delta^{5,24}$-cholestadien-3-β-ol) from cholesterol. Nevertheless, a limiting factor in the gas chromatography of sterols is the difficulty of obtaining satisfactory recovery with preparative columns.

The separation of sterol mixtures using thin-layer chromatography (TLC) has been recently reviewed by Copius-Peereboom and Beekes (5). The resolution obtained with silica gel or Kieselguhr is satisfactory for some sterols having different numbers of carbon atoms (6,7), while sterols differing only in respect to position and number of double bonds are slightly separated, but as either acetate (7) or trifluoroacetate derivatives (6).

Cholesterol and desmosterol can be separated by reversed phase TLC using either undecane (5,8) or paraffin oil (9) impregnated plates.

The application in column chromatography, introduced by de Vries (10), of a silver nitrate-coated adsorbent for the resolution of *cis-trans* and positional isomers of unsaturated fatty acids and their triglycerides, was extended to the TLC of sterols (7,11). Recently, Di Tullio et al. (12) obtained a good resolution of yeast cholesterol and desmosterol on thin-layer plates containing silver nitrate. Stokes et al. (13) separated sterols as their *p*-iodobenzoates on silicic acid: celite columns, and sterol acetates have been separated recently on silica gel columns by Klein and Szczepanik (14). However, all of these methods give only an incomplete resolution of the sterols.

In the present paper a separation of cholesterol-desmosterol mixtures using columns packed with Silica Gel G coated with silver nitrate on celite is described. Sterol mixtures obtained from livers and brains of rats treated with Triparanol were used for the separation. Desmosterol was recovered even from mixtures of brain sterols in which cholesterol represents 96% of the total sterols.

EXPERIMENTAL

Preparation of the sterol mixtures

Standards. Cholesterol (Merck, Darmstadt) and desmosterol (Merrell Co., Cincinnati) recrystallized in methanol.

Biological Materials. Groups of 6 Sprague-Dawley male rats weighing 250-300 g were injected i.p. for six days with 25 mg/kg of Triparanol [1 p(β-diethylaminoethoxy)-phenyl-1-(p-tolyl)-2(p-chlorophenyl) ethanol] (Wassermann S.p.A., Milan) in order to induce accumulation of desmosterol in the livers (1) and brains of the rats. The animals were sacrificed 12 hr after the last injection. The excised brains and livers were pooled, weighed and saponified in N KOH in 50% ethanol at 70C. Each rat liver weighed approximately 9 g and yielded about 25 mg of crude sterols per gram of tissue. Desmosterol represented 45% of these sterols, as calculated by gas-chromatographic analysis. Brains weighed 1.5 g and yielded about 13 mg of sterols per gram of tissue. Desmosterol represented 4% of the brain sterols, as calculated by gas chromatography. Aliquots of the original mixtures were purified, acetylated and used for column separations.

Sterol Purification. The unsaponifiable material was extracted in petroleum ether (bp, 60-80C). Three extractions were performed and the extracts combined, washed, and dried over anhydrous Na_2SO_4. The filtered extract

was evaporated under nitrogen and redissolved for purification by means of digitonide precipitation. The sterol digitonides were split in pyridine and the digitonin eliminated by precipitation in ethyl ether. The amount of total sterols present was determined by means of the colorimetric reaction of Zlatkis, Zak and Boyle (15), which gives the same extinction maximum (560 mμ) for both cholesterol and desmosterol.

Acetylation. Aliquots of the free sterols were dissolved in 20 parts of pyridine and 10 parts of acetic anhydride and allowed to stand for 24 hr under nitrogen, in the dark, at room temperature. The acetylated compounds were taken to dryness under vacuum and redissolved in hexane. Under the conditions described, no free cholesterol or desmosterol was detected upon TLC of 40 μg of the reaction mixture on Silica Gel G in ethyl ether-petroleum ether-acetic acid 30:70:1 (v/v/v). The chromatoplates were sprayed with 0.6 g $K_2Cr_2O_7$ in 100 ml of 55% (by weight) reagent grade H_2SO_4 and heated at 170C for 20 min before examination. Under these conditions, the minimal amount of free sterols which could be detected was 0.4-0.5 μg. The completeness of the acetylation was also controlled by means of gas chromatography, where impurities as low as 1% can be detected.

Separation procedure

Solvents. Benzene (B.D.H. Analar) washed with concentrated H_2SO_4, dried over $CaCl_2$, and distilled over sodium. Hexane (Merck), acetic anhydride and pyridine (Merck) freshly distilled. Acetic acid (Merck), petroleum ether (bp 60-80 c) and chloroform (B.D.H. Analar) not further purified.

Materials. Silica Gel G (Merck)—Celite 535 acid-washed. $AgNO_3$ (Carlo Erba, Milan).

Preparation of the Adsorbent. 10 g of Silica Gel G and 10 g of Celite were slurried with 10 ml of an aqueous solution of silver nitrate (40%). The mixture was heated at 110C for 48 hr. The adsorbent, ground in a mortar, was divided into portions of 1,5 g, reactivated at 110C for 1 hr and stored in covered flasks in a desiccator until used.

Preparation of the Column. Columns (25 cm in length, 9 mm I.D.), with a solvent reservoir and Teflon stopcock, were constructed of amber glass, in order to protect the light-sensitive adsorbent. A cotton plug was introduced at the bottom of the column, then the adsorbent in a hexane slurry was transferred into the column, and another plug placed on the top of the adsorbent. The adsorbent was washed with benzene and again with hexane. In standards separations, 1.5 mg of sterol acetates, dissolved in hexane, were applied on columns containing 1.5 g of adsorbent. Fractions of 5 ml each were collected throughout the chromatographic runs.

Identification and Purity Controls

The infrared spectra of cholesterol and desmosterol acetates were determined in Nujol. Melting points were determined by the capillary method. The mass spectra were determined by means of an LKB 9000 gas chromatograph-mass spectrometer.

Thin-Layer Chromatography. Five thin-layer plates were coated (250μ) with 25 g Silica Gel G in 50 ml of 15% aqueous $AgNO_3$ using a Chemetron (Milan) automatic spreader. Plates were activated at 110C for 1 hr and stored in an amber desiccator. A chloroform solution of 20-40 μg of sterol acetates was applied for each spot. Plates were developed with chloroform-petroleum ether (60-80C)—acetic acid 40:60:1 (v/v/v), which was found in preliminary experiments to be the best solvent system for the separation of the two sterol acetates. The plates were sprayed with 10% phosphomolybdic acid in ethanol and heated for 20 min at 170C.

Gas Chromatography. The cholesterol and desmosterol acetates were analyzed before and after column separation using a Fractovap Model C gas chromatograph (Carlo Erba Co.) equipped with a hydrogen flame detector and a glass column (2 m by 3 mm) packed with 1% CNSi (cyano-octyl methyl silicone, General Electric) on 100-120 mesh Gas-Chrom P, according to the method of VandenHeuvel and Horning (4). The carrier gas was nitrogen 1.7 kg/cm and the column temperature was 210C. The sterol acetates were identified using the method described by Fumagalli et al. (16), and the relative amounts of the two sterols in the original mixtures were calculated using cholestane as an internal standard.

RESULTS

Separation of cholesterol-desmosterol acetates with $AgNO_3$-silica gel columns was obtained as shown in Table I. Cholesterol was eluted with hexane-benzene 90:10; desmosterol with hexane-benzene 50:50 in the first experiments, but desmosterol may be also eluted with hexane-benzene 80-20. Fig. 1 shows a typical separa-

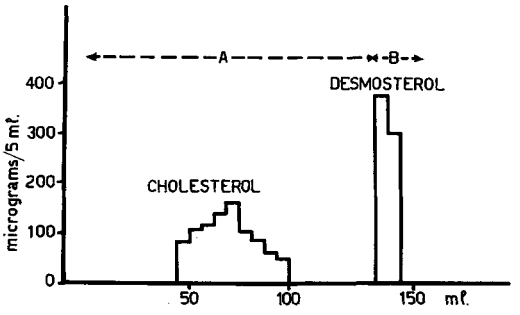

FIG. 1. Chromatography of a mixture of cholesterol-desmosterol acetates-solvent A: hexane-benzene 90:10-solvent B: hexane-benzene 80:20.

tion. It is possible to observe that after cholesterol elution, desmosterol is quickly obtained only when the solvent is changed. The ratio sterol-acetates/adsorbent was 1:2000 in the first column presented (Table I). Later on, a ratio of 1:1000 was found to give an equally complete resolution of the two sterols. Larger columns, containing 5 g of adsorbent, which could separate 5 mg of cholesterol-desmosterol acetates were also used.

The individual fractions were checked for purity by silver nitrate TLC and GLC, as shown respectively in a typical chromatoplate (Fig. 2) and gas chromatogram (Fig. 3). No overlapping of fractions was ever found by either GLC or TLC: impurities as low as 1% would have been detected by GLC even when 1 μg of sterol was applied to the column.

The acetate derivatives of the sterols extracted from the livers and brains of triparanol-treated rats were found to have Rf's on TLC, and retention times relative to cholestane on GLC, identical to those of cholesterol and desmosterol standards. The melting points and IR spectra of such acetates, after isolation from the silver nitrate columns, were in agreement with data reported in the literature. The melting point of desmosterol acetate (98C) was unchanged after a crystallization in methanol.

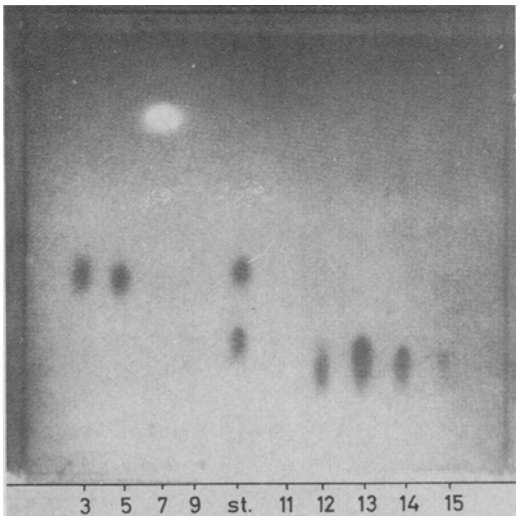

FIG. 2. TLC control of sterol acetates on silver nitrate-Silica Gel G. St.: 40 μg of the mixture of cholesterol (higher spot) and desmosterol (lower spot) acetates. No. 3 to 15: fraction numbers as in column IV (see Table I).

TABLE I

Isolation on Silver Nitrate Columns of Acetate Derivatives of Cholesterol and Desmosterol from Triparanol-Treated Rats

Column number	Solvent mixture hexane-benzene	Fraction No. (5 ml)	Sterol-acetates eluted	Applied μg	Found μg	Purity controls[d] TLC	GLC
I[a]	90 : 10	9 — 17	Cholesterol	825	825	p	..
	50 : 50	25 — 26	Desmosterol	675	675	p	..
II[a]	90 : 10	4 — 9	Cholesterol	825	820	p	p
	50 : 50	15 — 16	Desmosterol	675	665	p	p
III[a]	90 : 10	1 — 6	Cholesterol	....	....	..	p
	80 : 20	10 — 12	Desmosterol	....	....	..	p
IV[a]	90 : 10	1 — 6	Cholesterol	....	....	p	..
	80 : 20	12 — 16	Desmosterol	....	....	p	..
V[a]	90 : 10	5 — 13	Cholesterol	841	847	p	p
	80 : 20	20 — 24	Desmosterol	688	678	p	p
VI[a,b]	90 : 10	7 — 14	Cholesterol	....	....	p	..
	80 : 20	18 — 21	Desmosterol	....	....	p	..
VII[c]	90 : 10	5 — 15	Cholesterol	1920	1918	p	p
	80 : 20	20 — 24	Desmosterol	80	77	p	p

[a] Acetate derivatives of liver sterols.

[b] Column VI contained 5 g adsorbent; 5 mg of acetates were applied; 10 ml fractions eluted.

[c] Acetate derivatives of brain sterols.

[d] In purity controls each fraction eluted has been checked separately; p=pure to TLC and GLC.

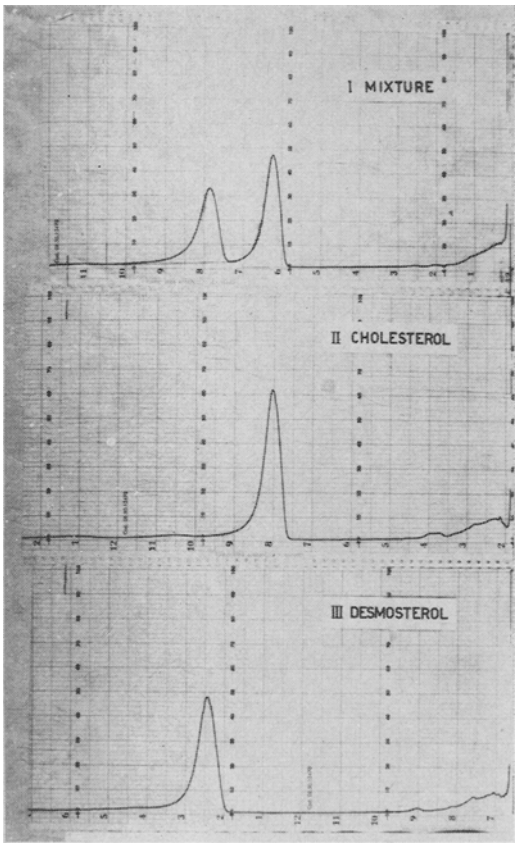

FIG. 3. GLC of sterol acetates on CNSi column:
I: cholesterol (peak 1) desmosterol (peak 2) mixture.
II: cholesterol eluted from $AgNO_3$ columns.
III: desmosterol eluted from $AgNO_3$ columns.

The molecular weights as determined by means of mass spectrometric analysis were 428 and 426, respectively, for cholesterol-acetate and desmosterol-acetate.

The percentage of desmosterol in the liver sterols of the rats treated with triparanol in this experiment was high. However, the relative amount of desmosterol naturally present in certain tissues or accumulating due to the action of hypocholesterolemic drugs, is often very small. Brain desmosterol, induced by triparanol treatment, was recovered by the present method, even from mixtures in which cholesterol represents as much as 96% of the total sterols (Table I, column VII).

The results show that selective and reversible binding properties of silver nitrate versus number and position of double bonds, definitely improve the separation of cholesterol and desmosterol obtained with silicic acid columns (13,-14). Moreover, the previous column methods do not give values demonstrating quantitative recoveries of small amounts of sterols (13,14). This is an essential condition for quantitative investigations of the last steps of cholesterol biosynthesis in liver and other tissues.

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Lipid Changes in Rat Brain During Maturation

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ABSTRACT

Changes in lipid class distribution occurring during brain maturation in rats have been quantitatively investigated using a combination of DEAE column chromatography and quantitative thin-layer chromatography based on photodensitometry.

Quantitative changes are presented for the single lipid classes in relation to brain weight, water content and total lipid variation at ages 2, 8, 16, 24, 37 and 50 days. The results indicate an accumulation of sphingolipids during brain maturation, particularly due to cerebrosides and sulfatides and a relative decrease of lecithin.

INTRODUCTION

THE IMPORTANCE of lipids in nervous structures has been long recognized and a large number of investigations have been carried out to detect and quantitate different lipid classes and to follow their changes during the critical period of brain maturation. The earliest work was summarized in 1962 by Sperry (1), who emphasized the difficulties of comparing results obtained by authors using different techniques.

Sometimes the values presented for the changes of individual lipid classes are in disagreement because of the lack of analytical tools for fractionating and quantitating single compounds. Very often different substances are included in a single class. This has been the case with the cephalins where the individual components (phosphatidyl ethanolamine [PE], phosphatidyl serine [PS])[1] were not measured separately (2-5). In many cases phosphatidyl inositol (PI) was included in the measurement of the cephalins. In the case of galactolipids it was found that they were not fractionated into cerebrosides, sulfatides and gangliosides (2-6). More recent data on cerebroside and cerebroside plus sulfatide variations during brain maturation are presented by Kishimoto and Radin (7,8).

The information available from the literature concerning brain lipids during development appears to be generally based on observations made prior to the advent of chromatographic techniques in the field of analytical lipid chemistry. Some of the uncertainties in estimating the major lipid components, that at the time could be measured only indirectly or were still ill-defined (as in the case of the so-called cephalins) are reflected in the difficulty of interpreting most data and suggest the importance of reinvestigating the problem on the basis of more adequate quantitative methods for separation of polar lipids (9-11). On the basis of these considerations, we have carried out a more extensive study of brain lipids using quantitative chromatographic procedures, with the aim of following the variations of the single lipid molecular species during maturation of the rat nervous tissues.

MATERIALS AND METHODS

Litters of six Sprague-Dawley rats were used for the experiments. Rats of 2,8,16,24, 37,50 days of age were killed by decapitation and brains immediately removed and pooled. The weights of the individual animals and their brains were measured and averaged. The combined brains were thoroughly ground in a mortar and two aliquots of approximately 250 mg from the homogeneous mass were used for determining the water content. The percentage of water was determined by drying the sample to constant weight over KOH in a desiccator under vacuum. The remaining part of the homogenate was stored at -20C until used.

Lipid extraction was carried out in a N_2 atmosphere with C/M 2/1 (9). Two-dimensional TLC was used routinely as the first step in examining each total lipid mixture (11). For TLC, Silica Gel G (Merck) was used as the adsorbent with C/M/NH_4OH 60/35/5 as the first developing solvent. Chromatograms were dried in a tank flushed with N_2 for 10 min and developed in the second dimension with C/A/M/HAC/H_2O 5/2/1/1/0.5. Spots were visualized by spraying the chromatograms with the sulfuric acid/potassium dichromate

[1]Abbreviations used: TLC, thin-layer chromatography; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; C, chloroform; M, methanol; NH_3, ammonia; A, acetone; HAc, acetic acid; PtE, petroleum ether; DE, diethylether.

charring reagent and heating at 180C for approximately 1 hr (10). A combination of DEAE column chromatography and TLC was used for the determination of the major lipid classes (10).

One gram of DEAE cellulose was used to prepare a 1 × 10 cm column (9) and 15 mg of total lipids were applied to the column. The elution scheme was as follows:

1) C/M 7/3 (cholesterol, cerebrosides, phosphatidyl ethanolamine, lecithin and sphingomyelin); 2) M (water-soluble nonlipid and oxidation products of phosphatidyl ethanolamine when present); 3) HAc (phosphatidyl serine and gangliosides); 4) M (to remove acetic acid); 5) C/M 4/1 + 20 ml/1 NH_4OH make 0.01 with respect to ammonium acetate (phosphatidic acid, sulfatide and phosphatidyl inositol). Columns were washed with methanol and finally with acetic acid, and could then be reused.

The fractions were evaporated in a rotary evaporator, transferred to glass stoppered tubes and dried again under a slight vacuum and under a stream of nitrogen. The solid residue was dissolved in 1 ml of C/M 2/1 and the solution was used for TLC determinations.

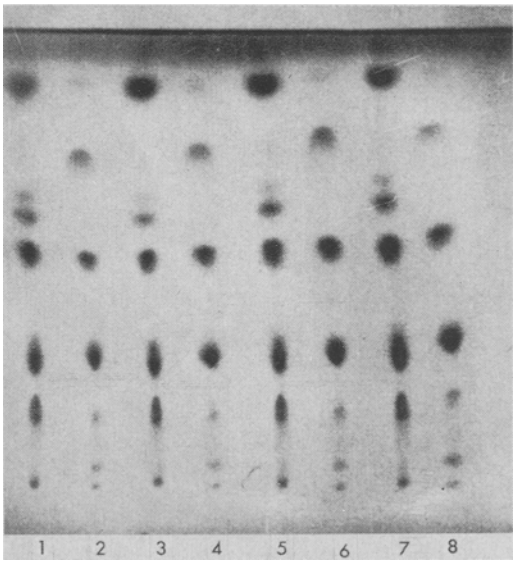

FIG. 1. Chromatogram prepared using Silica Gel G and chloroform/methanol/water 65/25/4 as solvent. Spottings 1,3,5,7 correspond to 40 γ each of the total brain lipid extract and spottings 2,4,6,8 are 30 γ each of mitochondrial lipid containing known amounts of cardiolipin, PE and lecithin (in order from top to bottom of the chromatoplate). The plate was used for the estimation of the amount of PE in the total extract.

Quantitative TLC

Transmission densitometry was used to measure the intensity of the charred spots and the amount of lipid present was determined by comparison with the charred spots of known amounts of authentic standards (10).

Spots were charred as previously described (10) and their optical densities were determined using model 520 Photovolt Transmission Densitometer.

Pure preparations of cholesterol, cerebrosides, sulfatides, sphingomyelin, fatty acids and a mitochondria lipid extract (kindly supplied by George Rouser, City of Hope Medical Center, Duarte, Calif.) containing known amounts of phosphatidyl ethanolamine, lecithin and cardiolipin (10) were used as standards.

Cholesterol, cerebrosides, PE and free fatty acids were determined by direct quantitative TLC of the total lipid extracts using C/M/H_2O 65/25/4 as developing solvent for cholesterol, cerebrosides and PE determinations; PtE/DE/HAc 70:30:1 for the quantitation of free fatty acids. This can be done since two-dimensional TLC excluded the overlapping of these lipid classes with other compounds on the chromatograms and also since it has been shown that the quantitation of spots directly on TLC, DEAE column chromatography, or a combination of the two procedures gives essentially the same results for the quantification of these compounds (10). In the case of PE the values obtained with direct TLC on the total lipid extract are higher than the values obtained by TLC of the C/M 7/3 fraction of the column. This is due to the elution of "altered" PE or, possibly, of decomposition products of PE in the subsequent fractions (10).

The values given for PE represent total PE, determined with direct TLC on the original extract (Fig. 1). Lecithin and sphingomyelin in the first fraction of the DEAE column and sulfatides eluted with C/M/NH_3/ammonium acetate were determined by quantitative TLC. PS was quantitated on the total lipid extracts after separation with the two-dimensional TLC system previously described, direct aspiration of spots by suction and phosphorus analysis without prior elution (12). Phosphorus values are multiplied by the factor 26.2 to express the results as amount of PS.

Cholesterol and desmosterol were separated using a gas chromatographic system with phenylmethyl silicone (PhSi) as stationary phase coated at 1% on 100-200 silanized Gas-

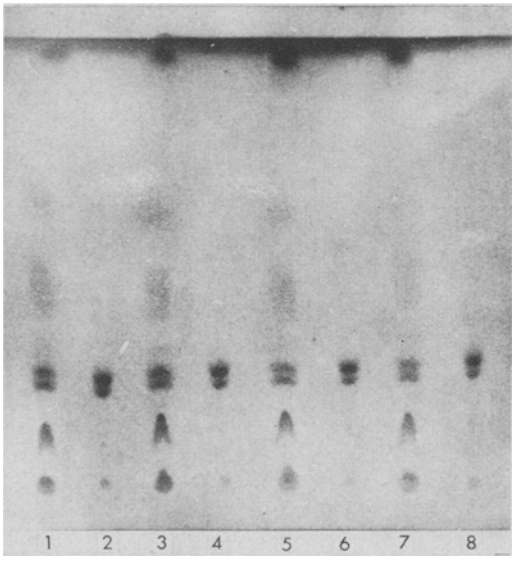

FIG. 2. Chromatogram used for the determination of the amount of sulfatide present in the C/M/NH_3 ammonium acetate fraction of the DEAE column. Spottings 1,3,5,7 are 10 μl each of the solution prepared from the lipid mixture eluted from the DEAE column. Spottings 2,4,6,8 correspond to 6 γ of sulfatide standard.

Chrom P. Analyses were carried out at 220C with N_2 as carrier gas with a flow of 30 ml/min. The gas chromatograph was a Fractovap model C (C. Erba, Italy) equipped with a flame ionization detector.

RESULTS

The changes in weight of body, whole brain and dry brain and total lipid per brain during development are shown in Table I. Body and brain weight values are averages obtained from 6 animals, while the water and lipid content were determined from a pool of 6 brains. During development, the increase of total lipid per brain shows a more uniform trend and greater variation (twelvefold increase) than the increase in brain weight (fivefold) and percentage of brain lipids. Furthermore, brain weight shows a slight decrease between the 16th and 24th days of age, as observed also by other authors (13), but total lipid content increases uniformly.

The variations of brain sterols with age are shown in Table II. Total sterols do not vary appreciably as percentage of total lipids. The sterol as percentage of the dry weight increases during the first 2 weeks and then remains relatively constant.

Cholesterol, as percentage of the fresh weight, shows a more prolonged increase since brain weight also increases. The total amount of sterol shows instead a constant gain. The relative amount of desmosterol is greatest at birth and then decreases uniformly. The concentrations expressed as percentage of fresh and dry weight are maximal at 1 week and then decrease sharply. The total amount of desmosterol per brain is appreciable even after 50 days.

Tables III and IV show the variations of polar lipid class composition during brain maturation. The values are expressed as percentage of total lipids. In Table III it is apparent that PE and PS remain practically constant in relation to total lipid during development. On the other hand, lecithin decreases, while the ratio lecithin/PE decreases from 1.21 to 0.63 and the other lipids show a continuous increase in percentage. The increase is greatest for cerebrosides and sulfatides and somewhat less for sphingomyelin.

TABLE I
Brain Weight and Total Lipids During Growth[a]

Age, days	Body wt, g	Brain wt, mg	Mg dry wt per brain	Dry wt, as % of fresh wt	Total lipids as % of dry wt	Total lipids as % of fresh wt	Mg of lipids per brain
2[b]	11	364	43.68	12	28.7	3.45	12.5
	9.3 ± 0.21	364 ± 4.7	45.87	12.6	27.0	3.43	12.4
8	17 ± 0.6	737 ± 9.3	93.34	12.8	28	3.50	26.0
16	47	1440	252	17.5	34.8	6.30	90.7
24	72	1400	291.2	20.8	40.6	8.50	118.0
37	142 ± 6.4	1690 ± 30	380.25	22.5	36.4	8.20	138.0
50	203 ± 6.4	1789 ± 29	411.47	23.0	37.0	8.50	152.0

[a] Litters of 6 animals each.
[b] Two different groups of animals were analyzed at 2 days of age.

TABLE II
Brain Sterols During Growth[a]

Age, days	% Total lipids	% Desmosterol, % of total sterols	% Fresh wt			% Dry w			Mg/brain		
			Total sterols	Ch.	D.	Total sterols	Ch.	D.	Total sterols	Ch.	D.
2	13.4	24.5	0.464	0.356	0.112	3.99	3.0	1.0	1.70	1.30	0.41
	17.0	24.5	0.578	0.437	0.107	4.60	3.5	1.1	2.10	1.58	0.51
8	19.7	23.5	0.695	0.532	0.163	5.56	4.2	1.3	5.10	3.91	1.20
16	22.3	4.5	1.410	1.340	0.063	8.00	7.7	0.4	20.30	19.39	0.91
24	18.5	1.3	1.560	1.530	0.023	7.50	7.4	0.1	21.80	21.51	0.28
37	21.5	1.3	1.760	1.750	0.022	7.85	7.8	0.1	30.00	29.61	0.39
50	21.2	1.2	1.800	1.770	0.021	7.85	7.7	0.1	32.10	31.71	0.38

[a] Ch. = Cholesterol; D. = Desmosterol

TABLE III
Lipid Classes as Percentage of Brain Total Lipids During Growth[a]

Age, days		Total sterols	Lec	PE	Lec/PE	PS	Sph	Cer	Su	FFA
2	A	13.4	26.5	22.4	1.21	*	—	—	—	*
	B	17.0	33.9	28.0	1.21	4.25	—	—	—	*
8		19.7	26.9	22.3	1.21	*	0.72	—	—	3.4
16		22.3	22.8	26.6	0.86	5.75	2.25	4.25	0.81	2.9
24		18.5	24.3	31.8	0.76	*	2.15	6.15	1.15	2.66
37		21.5	18.5	24.3	0.76	*	3.00	11.0	4.08	2.7
50		21.2	16.5	26.3	0.63	5.60	2.51	11.0	3.57	2.7

[a] For abbreviations and symbols, see Table IV.
*Not determined.

TABLE IV
Brain Lipid Classes as Percentage of Fresh and Dry Weight

	Total sterols		Lec		PE		PS		Sph		Cer		Su		FFA	
Age	% FW	% DW	% FW	% DW	% FW	% DW	% FW	% DW	% FW	% DW	% FW	% DW	% FW	% DW	% FW	% DW
2 A	0.46	3.99	0.90	7.50	0.78	6.48	*	*	—	—	—	—	—	—	*	*
2 B	0.58	4.60	1.15	9.15	0.96	7.60	0.14	1.15	—	—	—	—	—	—	*	*
8	0.70	5.50	0.95	7.50	0.79	6.20	*	*	0.02	0.23	—	—	—	—	0.12	0.93
16	1.41	8.00	1.44	8.10	1.68	9.60	0.36	2.05	0.14	0.81	0.27	1.53	0.05	0.29	0.18	1.03
24	1.56	7.50	2.05	9.22	2.67	12.90	*	*	0.18	0.87	0.52	2.50	0.09	0.47	0.22	1.07
37	1.76	7.85	1.50	6.70	2.00	8.75	*	*	0.25	1.10	0.90	4.00	0.34	1.48	0.23	1.02
50	1.80	7.85	1.41	6.10	2.23	9.75	0.47	2.05	0.21	0.92	0.93	4.10	0.30	1.32	0.23	1.02

Lec lecithin
PE phosphatidyl ethanolamine
PS phosphatidyl serine
Sph sphingomyelin
Cer cerebrosides
Su sulatides
FFA free fatty acids

* not determined
— traces

FW fresh weight
DW dry weight

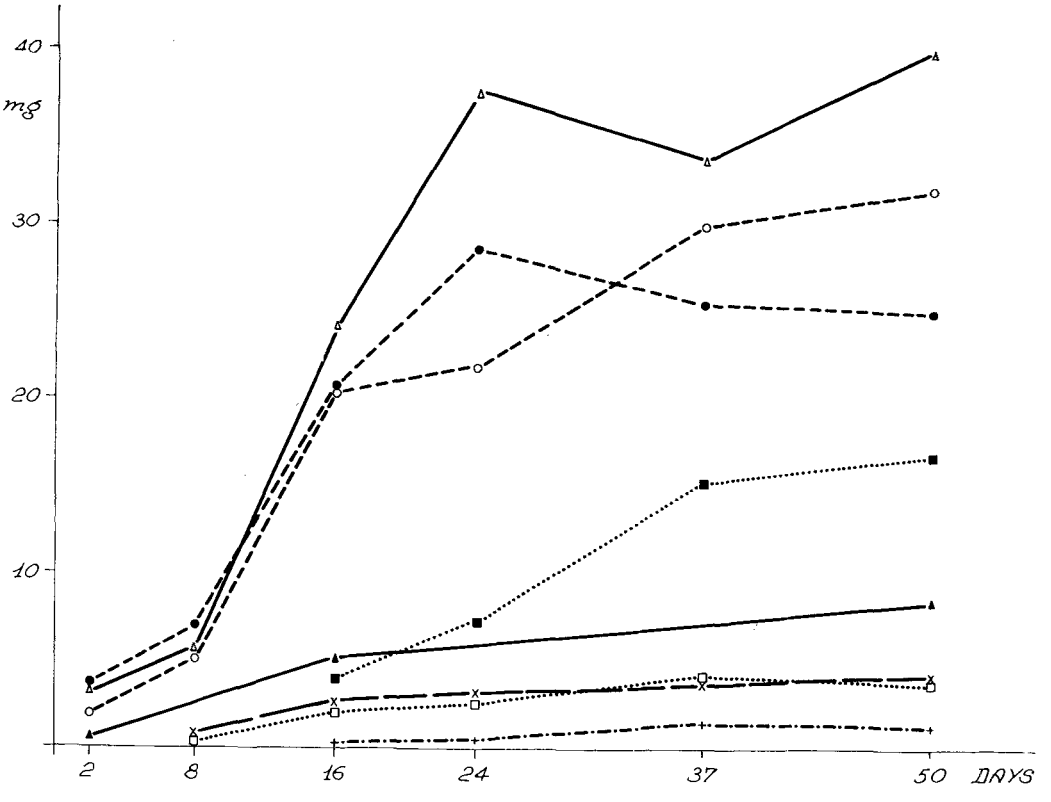

FIG. 3. Lipid classes, mg/brain.

Table IV shows the variations of lipid classes expressed as percentage of fresh and dry weight. Lecithin and PE reach the maximal concentration after 3 weeks (twofold and threefold, respectively on the basis of wet weight) and similar changes are observed when the concentrations are measured on the basis of dry weight. PS shows a uniform increase on a wet weight basis, but the concentration based on a dry weight is constant after two weeks of age. The same is true for total sterols.

Sphingomyelin increases ten times in concentration on a fresh weight basis, considerably less (four- to fivefold) on a dry weight basis. Cerebrosides and sulfatides appear later than phospholipids and their accumulation is more pronounced, showing the same trend when expressed on a wet or dry weight basis. The variations of the single lipid classes when expressed as milligrams of lipids per brain are presented in Fig. 3. Lecithin and PE reach a maximum at the end of the third week of age, while sphingomyelin, sulfatides and cerebrosides are still increasing in concentration in the brains of animals at 50 days of age.

DISCUSSION

The importance of lipids of the brain during the myelination process is apparent when it is

noted that the total lipid content increases twice as fast as the weight of the brain.

The rate of accumulation of total lipids is maximal during the first 3 weeks (100 mg) and decreases later on (30 mg during the following 3 weeks). However, total brain weight and lipids are still increasing after 2 months (7). Since myelination appears to be complete in rat brain at roughly 50 days of age as judged from histological observations (14), some authors (7) suggest that after this point the deposition of nonmyelin lipids takes place. The relatively high percentages of PE and lecithin, and the low values of sphingomyelin, and cerebrosides in 50-day-old rat brains in comparison with the values found in adult brains of other species, beef and human (9,15), may indicate instead that lipid deposition after 50 days of age is not histologically detectable and that morphological observations may not be the most adequate method to follow brain maturation.

Changes in the percentage composition of the different lipid classes during brain growth, suggest some consideration as to the role they may play in the process of maturation. Since the number of cells and cell types in brain is assumed to remain constant during maturation and the chemical composition of well-defined and stable elements (e.g., myelin) is assumed to remain qualitatively and quantitatively constant, variations of chemical compounds should be related to the existence of a compartmentalized development, i.e. appearance of new specialized structures. From the data available in the literature it appears that total phosphatides show a slight increase in concentration during brain maturation in all species investigated (2,3). However, no recent data are available on the changes in polar lipid distribution. Lecithin has mostly been estimated by difference, on the basis of choline and sphingomyelin determinations (2-4, 6). The findings for both of these lipid classes are variable and sometimes inconsistent, but it is generally reported that lecithin concentration does not change while sphingomyelin shows a sharp increase. Even fewer data are available on PE and PS developmental changes in brain, since most papers do not present separate determinations of these two classes. According to Folch (5) PS follows a trend of accumulation similar to that of lecithin, while PE shows a rate of deposition similar to that of sphingomyelin in the mouse brain.

Although some of the previous data suggest trends of variations in the biochemistry of brain lipids during early maturation, they do not present adequate quantitative information on the concentration values (expressed on a wet and dry weight basis) of each major single lipid and on the relative changes in rat brain. Our values for cerebroside concentrations and their variations are consistent with those found by Radin et al. (7,8).

No previous information has been reported for sulfatide concentrations and their changes in developing rat brain.

The changes for sphingomyelin are greater than those previously reported for mice on a wet weight basis (5), but the results cannot be compared because of the difference in the animal species used. In conclusion, sphingolipids (cerebrosides, sulfatides and sphingomyelin), not present at birth, appear in detectable amounts after 3 weeks and show a pronounced increase in percentage (percentage of total lipids or percentage of dry or fresh weight) up to 50 days of age.

Other major compounds are already present at high concentrations at birth, in respect to total lipids (cholesterol, lecithin, phosphatidyl ethanolamine and phosphatidyl serine), while they reach the maximal concentrations in respect to fresh or dry brain weight, after approximately 3 weeks.

Lecithin shows the lowest increase in concentration on a fresh weight basis, while there is a decrease in concentration on a dry weight basis. This is in contrast with findings by other authors (5) showing for PS an increase lower than that of lecithin and for PE an accumulation similar to that of sphingomyelin. Cholesterol accumulates faster than phosphatides with the exception of sphingomyelin. The procedure used opens the possibility of obtaining a quantitative determination of the single lipid components in brain of growing rats and of following changes in total and relative amounts. This approach gives a sensitive way to detect possible hormonal and dietary effect on brain lipid deposition and brain maturation.

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SHORT COMMUNICATIONS

Reaction of Oxidizing Lipids with Ribonuclease

THE OXIDATION of polyunsaturated fatty acids produces several 2-thiobarbituric acid-reactive substances (TBRS) of which malonaldehyde appears to be the principal one (1). Kwon et al. (2) have described the reactivity of malonaldehyde (MA) with food constituents and the isolation of a myoglobin-TBRS reaction product from frozen tuna red muscle. Packer et al. (3) have reviewed the subject of oxidation of polyunsaturated fatty acids as a mechanism of biological membrane disruption. Considerable importance has been placed on the free radical mechanism of protein damage resulting from the formation of free radicals, lipid hydroperoxide and other, during oxidation. While free radicals arising from lipid oxidation may react with membrane proteins, we wish to point out that the TBRS, also products of the oxidation, are in themselves reactive and that TBRS react with proteins and cause their polymerization.

The oxidation of linolenic acid (0.46 g, Hormel Foundation) was allowed to proceed spontaneously at 30C in the presence of 0.22 g of bovine pancreatic ribonuclease (RNase, Sigma Chemical Co.) in 2.5 ml of 0.1 M phosphate buffer, pH 7.6 in a standard Warburg apparatus. The reaction was stopped when the oxygen uptake, as measured manometrically, reached an oxygen-to-linolenic acid molar ratio of 0.2 The reaction mixture was extracted exhaustively with cold chloroform and petroleum ether to remove free lipid components. A small amount of insoluble protein was removed by centrifugation. The resulting solution was dialyzed against glass distilled water for 72 hr at 4C to remove free TBRS. The TBRS reacted with the RNase could only be released by hydrolysis in hot 1 N HCl. The protein concentration was determined by the biuret reaction and the TBRS by the 2-thiobarbituric acid reaction. Two milliliters of the yellowish dialyzate containing 43 mg of RNase was analyzed by gel filtration on a 2 × 98 cm column of Sephadex G-100 as previously described (2). Elution patterns were determined by measuring the absorbance continuously at 254 mμ with an ISCO ultraviolet photometer.

RNase (0.340 g) was also reacted with 20 mM MA in 5 ml of 0.1 M phosphate buffer, pH 7.6, at 30C for 4 hr. Two milliliters of the reaction mixture were chromatographed as above.

The relationship between the elution volume and the molecular weight of the polymers was estimated from a plot of the log of the molecular weight of known proteins vs. elution volume. Blue dextran 2000 (Pharmacia Co.) was used to determine the void volume; RNase, trypsin, pepsin, ovalbumin (Sigma), and aldolase were used to calibrate the column.

Figure 1A illustrates the gel filtration of unreacted RNase, while Figure 1B illustrates that of the RNase reaction product isolated from the oxidized lipid mixture. The elution volumes of the three components correspond to molecular weights of 14,000, 28,000, and 42,000, as would be expected for the monomer, dimer, and trimer of RNase.

While the unreacted RNase contained no aggregates, RNase reacted with TBRS was mostly dimer. Some polymers of molecular weight greater than 42,000 were also present. The main component (28,000 mol wt) of the TBRS-reacted RNase contained 0.05 moles of TBRS per mole of RNase, calculated as MA equivalents. No free TBRS were observed.

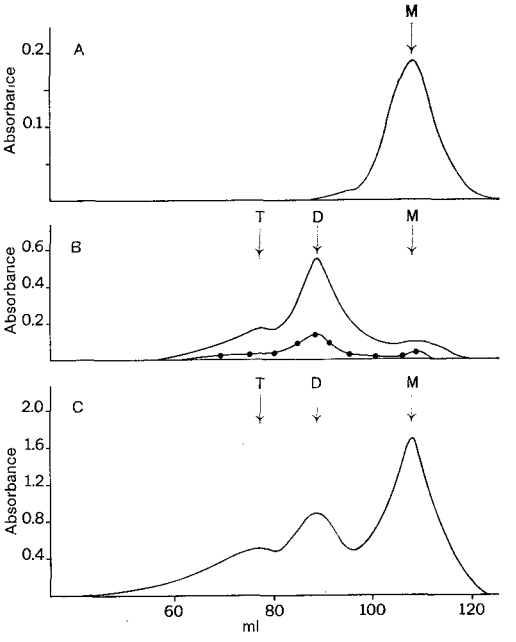

FIG. 1. Gel filtration of RNase reaction products: *A*, unreacted RNase; *B*, RNase reacted with oxidizing linolenic acid as described in the text; *C*, RNase reacted with MA. Solid lines represent the protein absorbance measured continuously at 254 mμ; circles the absorbance of the TBA reaction of the fractions measured at 532 mμ.

Figure 1C illustrates the gel filtration of RNase reacted with MA alone. Components corresponding to molecular weights of the monomeric, dimeric, and trimeric RNase were found. A small amount of protein of molecular weight greater than 42,000 was also present.

Since the principal TBRS is malonaldehyde (1), it is probable that malonaldehyde accounts for most of the TBRS reacted with RNase. Malonaldehyde alone at 20 mM concentration produced polymeric forms of RNase similar to those observed on lipid oxidation. If all of the dimer were the result of the biomolecular reaction with malonaldehyde, a malonaldehyde content of 0.5 moles per mole of RNase would be expected. In the present experiment, the maximum ration found was 0.05 moles TBRS per mole RNase, indicating that dimers produced by other reactions are present. The reaction of proteins with TBRS, and particularly malonaldehyde, may thus account for some of the biological consequences currently ascribed to the free radicals produced by oxidation of polyunsaturated fatty acids (3). TBRS reacted proteins may be of biological importance as a possible source of the "age pigment" as discussed by Bjorksten (4).

ACKNOWLEDGMENT

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Quantitative Separation of C-27 Sterol Precursors of Cholesterol

IN A RECENT PAPER from this laboratory (1) the quantitative separation of cholesterol (Δ5-cholesten-3β-ol) and desmosterol (Δ5,24-cholestadiene-3β-ol), using columns packed with Celite-Silica Gel G impregnated with silver nitrate, was described. The reproducibility of this method prompted us to determine whether or not more complex sterol mixtures could be separated completely using the same technique.

Sterol mixtures used were obtained from livers of rats treated for 7 days with 20 μmoles/kg (i.p.) Triparanol [1-p(β-diethylaminoethoxy)-phenyl-1-(p tolyl)-2(p-chlorophenyl) ethanol] (Wassermann S.p.A.) and 10 μmoles/kg (s.c.) AY 9944 [*trans*-1-4-*bis* (2-dichlorobenzylaminomethyl) cyclohexane dihydrochloride] (Ayerst, McKenna and Harrison, Ltd.). It has been demonstrated that the combined administration of these two drugs induces an accumulation of desmosterol, Δ 5,7-cholestadiene-3β-ol (7-dehydrocholesterol), and Δ 5,7,24-cholestatriene-3β-ol (7-dehydrodesmosterol) in liver (2,3).

The extraction, digitonin purification, determination of total sterols, and acetylation procedure were performed as previously described (1). The completeness of the acetylation reaction was controlled with GLC and TLC tests and the relative amounts of the acetylated sterols present in the mixture were determined by GLC using cholestane as an internal standard (4).

The preparation and activation of the adsorbent and column chromatography procedure was essentially the same as that reported previously (1), except that a larger amber column was used (40 cm long, 9 mm I.D.). A ratio of 1:1000 was maintained between the sterol acetates and the adsorbent. A slurry of the activated adsorbent (1 g Silica Gel G, 1 g Celite, 0,4 g $AgNO_3$) in hexane was added to the column, and the adsorbent washed with benzene and again with hexane. Sterol acetates (2 mg) were added to the column in hexane and separated according to the elution gradient given in Table I. The elution sequence is cholesterol, desmosterol, 7-dehydrocholesterol and 7-dehydrodesmosterol. Certain precautions should be taken to prevent decomposition of the sterols, especially of 7-dehydrocholesterol and 7-dehydrodesmosterol, which contain unstable conjugated bond systems. Manipulations before and after application of the sample to

Figure 1C illustrates the gel filtration of RNase reacted with MA alone. Components corresponding to molecular weights of the monomeric, dimeric, and trimeric RNase were found. A small amount of protein of molecular weight greater than 42,000 was also present.

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Quantitative Separation of C-27 Sterol Precursors of Cholesterol

IN A RECENT PAPER from this laboratory (1) the quantitative separation of cholesterol (Δ5-cholesten-3β-ol) and desmosterol (Δ5,24-cholestadiene-3β-ol), using columns packed with Celite-Silica Gel G impregnated with silver nitrate, was described. The reproducibility of this method prompted us to determine whether or not more complex sterol mixtures could be separated completely using the same technique.

Sterol mixtures used were obtained from livers of rats treated for 7 days with 20 μmoles/kg (i.p.) Triparanol [1-p(β-diethylaminoethoxy)-phenyl-1-(p tolyl)-2(p-chlorophenyl) ethanol] (Wassermann S.p.A.) and 10 μmoles/kg (s.c.) AY 9944 [*trans*-1-4-*bis* (2-dichlorobenzylaminomethyl) cyclohexane dihydrochloride] (Ayerst, McKenna and Harrison, Ltd.). It has been demonstrated that the combined administration of these two drugs induces an accumulation of desmosterol, Δ 5,7-cholestadiene-3β-ol (7-dehydrocholesterol), and Δ 5,7,24-cholestatriene-3β-ol (7-dehydrodesmosterol) in liver (2,3).

The extraction, digitonin purification, determination of total sterols, and acetylation procedure were performed as previously described (1). The completeness of the acetylation reaction was controlled with GLC and TLC tests and the relative amounts of the acetylated sterols present in the mixture were determined by GLC using cholestane as an internal standard (4).

The preparation and activation of the adsorbent and column chromatography procedure was essentially the same as that reported previously (1), except that a larger amber column was used (40 cm long, 9 mm I.D.). A ratio of 1:1000 was maintained between the sterol acetates and the adsorbent. A slurry of the activated adsorbent (1 g Silica Gel G, 1 g Celite, 0,4 g $AgNO_3$) in hexane was added to the column, and the adsorbent washed with benzene and again with hexane. Sterol acetates (2 mg) were added to the column in hexane and separated according to the elution gradient given in Table I. The elution sequence is cholesterol, desmosterol, 7-dehydrocholesterol and 7-dehydrodesmosterol. Certain precautions should be taken to prevent decomposition of the sterols, especially of 7-dehydrocholesterol and 7-dehydrodesmosterol, which contain unstable conjugated bond systems. Manipulations before and after application of the sample to

TABLE I
Column Chromatography of Acetate Derivatives of Sterols from Livers of Rats Treated with Triparanol and AY 9944

Solvent mixture, hexane-benzene	Fraction N° (10 ml fractions)	Sterol acetates eluted	Applied μg	Found μg
90- 10	2-12	Cholesterol	1449	1473
80- 20	22-40	Desmosterol	376	377
60- 40	48-52	7-Dehydro-cholesterol	82	86
100	58-76	7-Dehydro-desmosterol	140	138

the column should be performed as much as possible in the absence of heat and light, and in a nitrogen atmosphere. Also the runs are performed under nitrogen. The individual fractions (10 ml) were analyzed by silver nitrate-TLC and GLC using the same experimental conditions described for cholesterol and desmosterol in the previous paper (1). Comparisons with recrystallized cholesterol, desmosterol, and 7-dehydrocholesterol (Calbiochem.) standards, showed that the sterols were quantitatively eluted in pure form. The structure of 7-dehydrodesmosterol was determined, and the identity of the other sterols confirmed, by means of an LKB 9000 gas chromatograph-mass spectrometer. The recovery of the sterols was determined on the pooled fractions of each sterol acetate on GLC, using cholestane as the internal standard (4). Sterols differing only in double bond position, e.g. desmosterol and 7-dehydrocholesterol, are completely resolved by the present method. This separation is particularly useful because these two sterols are poorly separated on GLC and a preparative separation on TLC with $AgNO_3$ impregnated plates can not be easily applied because of the chemical instability of 7-dehydrocholesterol when exposed to air and light.

ACKNOWLEDGMENTS

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[Received Aug. 31, 1966]

Detection of Phthalate Esters as Contaminants of Lipid Extracts from Soil Samples Stored in Standard Soil Bags

DURING THE COURSE of a study of soil lipids we became aware of a major component that proved to be one of several contaminants. Soil samples from numerous sites were collected in standard plastic-lined canvas bags (Soiltest, Inc., Catalog No. LT-93, 2205 Lee St., Evanston, Ill.). Lipids were extracted with chloroform/methanol 2/1 and examined by thin-layer chromatography (TLC) in a variety of solvent systems. TLC showed that a major component which moved like a triglyceride was present in all but one of the soil extracts. This major component was isolated and characterized immediately.

Preparative TLC was carried out on silicic acid mixed with 10% magnesium silicate using hexane/ether 60/40 as the developing mixture. The major component was localized by spraying with water and then eluted with chloroform. This spot represented from 50-80% of the weight of the various soil extracts. Using the potassium bromide pellet technique, identical infrared spectra were obtained from the major component of various soils. The typical spectrum (Fig. 1), while similar to that of a triglyceride, contained bands at 1070, 1130 and 1280 cm^{-1} characteristic of phthalate esters. Dioctyl, dinonyl, didecyl and other

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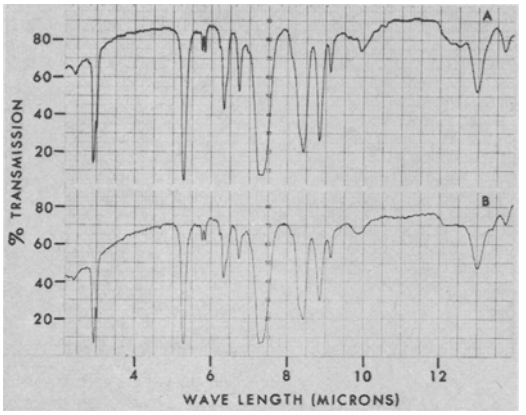

FIG. 1. *A*, infrared spectrum (1.82% in potassium bromide) of the phthalate ester isolated from a soil sample; *B*, authentic didecylphthalate.

phthalate esters migrated on TLC to the same position as that of the soil sample spot. The migration of dioctyl phthalate is shown in Fig. 2.

The substance isolated from the soil samples was hydrolyzed in 2 N methanolic HCl

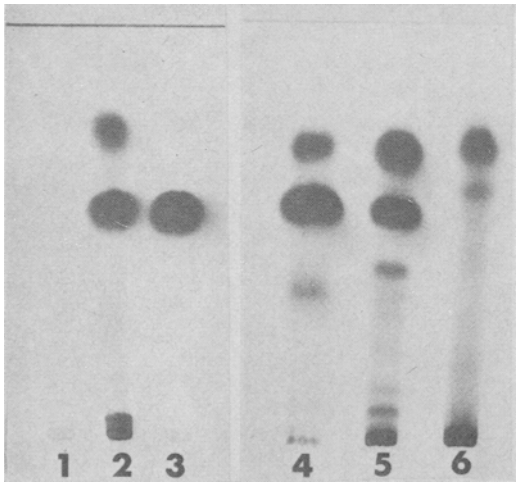

FIG. 2. One-dimensional TLC with hexane/diethyl ether 60/40 as solvent and char spray. 1) 200 μg of soil 73 (clean sand) extract uncontaminated; 2) 200 μg of soil 73 extract after being contaminated by material from the soil bag; 3) 200 μg of dioctylphthalate; 4) 300 μg of chloroform/methanol 2/1 extractable material from a standard soil bag liner showing a large amount of phthalate ester; 5) 300 μg of extract of soil 206 after being shaken in a standard soil bag where phthalate esters entered as contaminants (compare with application 6); 6) 300 μg of extract of soil 206 not stored in a soil bag and free of phthalate esters (compare with application 5).

to yield phthalic acid and a mixture of alcohols. The alcohols were identified by gas chromatography using Porapak Q at 230C as the stationary phase with helium as the carrier gas at a flow rate of 45 ml/min. Retention times which corresponded to those of *n*-octyl and *n*-decyl alcohols were obtained from the hydrolysate of the spot isolated from the soil samples. The substance from the soils which migrated like a triglyceride on TLC was thus identified as a mixture of dioctyl and didecyl phthalates.

It was evident that this substance represented an artifact of the standard processing procedures for soils, even though phthalic acid esters have been reported in petroleum (1) and in a uranium asphaltite (2). The obvious source of contamination was the standard plastic-lined soil bag. Extraction of both the bag liner and a clean sand sample which had been shaken in a bag yielded material with TLC behavior (Fig. 2) and an infrared spectrum identical to that from the soils. Freshly obtained soils not placed in the standard bags showed no phthalate ester contamination (Fig. 2).

Phthalic acid esters as contaminants in lipid analysis must be added to the list of potential contaminants recently considered (3) and the hydrocarbon contaminant of TLC adsorbent reported by Ma (4). Soil samples collected for lipid analysis should be collected in glass or other inert containers. Suitable nonplasticized bag liners are those made from Aclar (available from Specialty Converting, Inc., El Monte, Calif.).

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Rapid Determination of Lipids Containing Free Amino Groups with Trinitrobenzene Sulfonic Acid Reagent

THE DETECTION AND ANALYSIS of lipids containing free amino groups by rapid, reliable screening procedures for column chromatographic fractions are desirable. Reaction with ninhydrin has been used for this purpose (1,2). Ninhydrin reagents, however, are difficult to use since reproducible color yields are obtained only with very careful control of all conditions and color yields vary widely with different substances. In contrast, reactions with trinitrobenzene sulfonic acid (TNBS) yield uniform, reproducible and stable color intensities for free amino groups of a variety of substances, both lipid and nonlipid. The procedure as published by Satake (3) for amino acids can not be applied to lipids because of their hydrophobic nature. The present report describes a TNBS procedure for the quantitative analysis of intact amino-containing lipids.

Samples were dissolved in chloroform/methanol 2/1 and an aliquot containing 0.05–0.40 micromoles of lipid amine was pipetted into a 12-ml graduated centrifuge tube. Enough chloroform/methanol 2/1 was added to bring the volume to 1 ml. One milliliter of neutralized Hyamine hydroxide was added followed by 1 ml of 0.1% TNBS (Nutritional Biochemicals Corp., Cleveland, Ohio) in distilled water. The Hyamine hydroxide [p-(diisobutylcresoxyethoxyethyl) dimethylbenzyl ammonium hydroxide] (Packard Instrument Co., La Grange, Ill.) was neutralized to pH 8.0 with glacial acetic acid and diluted to 0.5 M with methanol. After mixing thoroughly, the samples were incubated at 40C for 2 hr in a water bath covered with aluminum foil to exclude light. One milliliter of methanolic 1 N hydrochloric acid (8.33 ml concentrated hydrochloric acid diluted to 100 ml with absolute methanol) was added to quench the unreacted TNBS color and the volume was made up to 5 ml with methanol. The color intensity was read at 340 mμ using a reagent blank as zero.

The standard curve for ethanolamine (Fig. 1) obtained with the original Satake (3) procedure and by the present procedure are identical even though one reaction (solid circle) is carried out using the procedure for chloroform/methanol soluble compounds.

The absorption curves of the TNBS reaction products for serine and ethanolamine as well as phosphatidyl serine and phosphatidyl ethanolamine (Fig. 2) are superimposable. All reaction products absorb at the same maximum

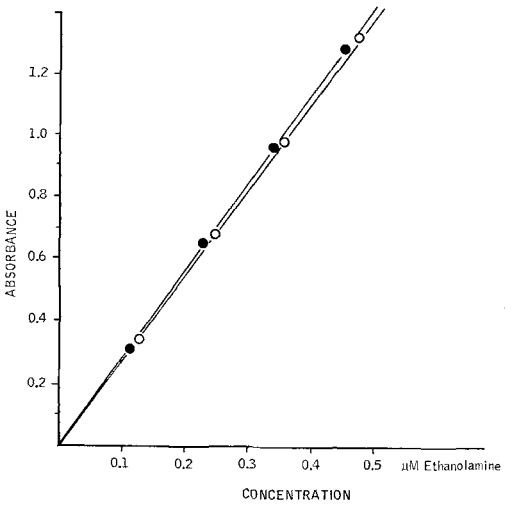

FIG. 1. Standard curves for ethanolamine using the procedure of Satake (3) (○), and the methods presented in this paper (●).

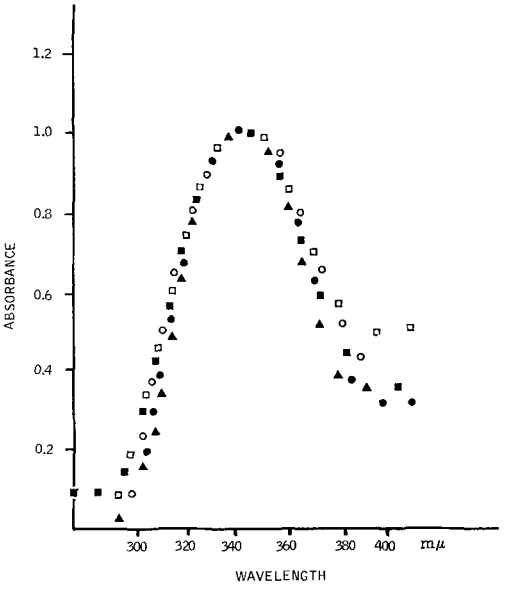

FIG. 2. Absorption curves for TNBS reaction products. Ethanolamine (○), serine (□) by the method of Satake (3). Ethanolamine (●), phosphatidylethanolamine (▲) and serine (■) by this method.

(340 mμ). The present method gives comparable color intensity yields and the same product absorption curves for lipid-like materials as does the Satake (3) procedure for water-soluble amino acids. This procedure has been employed for monitoring silicic acid column fractions for phosphatidyl ethanolamine and phosphatidyl serine and the results obtained agreed with those obtained by the method of Lea and Rhodes (2).

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[Received Sept. 15, 1966]

LETTERS TO THE EDITOR

A Note on the Fatty Acids Present in Oilseed Phospholipids

Sir: Hilditch (The Chemical Constitution of Natural Fats, 4th Ed., 1964, Chapman and Hall, London, p. 346) stated: "All the acids present in any seed glyceride are also found in the corresponding seed phosphatide." He further stated that "Seed phosphatides may contain characteristic although minor proportions of highly unsaturated C_{20} and C_{22} acids, which are not present in the corresponding glycerides." In both, palmitic acid is usually the most abundant saturated acid, with stearic and arachidic acids present to lesser extents. Again, in both, oleic and linoleic acids are usually the chief unsaturated components, with the latter often predominating in the phospholipids.

Recent evidence has cast doubts on these notions. James et al. (Biochem. J. *95*, 448, 1965) cited an unpublished observation of Nichols et al. that ricinoleic acid is absent in castor seed phospholipids. Our own studies (Paulose et al., Indian J. Chem., in press) at about the same time using thin-layer chromatography, (TLC) and gas-liquid chromatography (GLC) of the fatty acids of castor seed phospholipids (purified chromatographically and found to consist mainly of glycerophospholipids) revealed for the first time the presence (as acids wt %) of palmitic (27.7), stearic (12.9), oleic (18.5) and linoleic (33.2) as the major components accompanied by small percentages of lauric (0.8), myristic (0.3), arachidic (1.5) and arachidonic (3.9) acids. Ricinoleic acid, which constitutes 90% of the castor oil glycerides, is completely absent from castor seed phospholipids.

Rape and mustard seed oils also contain a characteristic fatty acid, viz. erucic, to the extent of 40-50%. While Hilditch and Pedelty (Biochem. J. *31*, 1964, 1937) reported 22% of erucic acid in the cephalin fraction of the phospholipids, recent work indicates its complete absence from the total phospholipids. Heiduschka and Neumann (J. Prakt. Chem. *151*, 1, 1938; C. A. *32*, 7076, 1938) were of this view, and the recent work of Weenink and Tulloch (JAOCS *43*, 327, 1966) on individual separated phospholipid classes using GLC provides further and more accurate confirmation. Our simultaneous studies (Paulose et al., J. Chromatog. *21*, 141, 1966) on the fatty acids of Indian mustard seed phospholipids using a sensitive TLC method which separates individual saturated and unsaturated acids, failed to provide any evidence for the presence of erucic acid. Thus ricincleic acid and erucic acid, which are present in castor and rape seed oils to the extent of 90 and 50%, respectively, are conspicuously absent from the phospholipids.

These two cases are particularly striking since easily recognizable fatty acids are involved. They clearly show that the fatty acids of phospholipids need not be qualitatively the same as those of corresponding glycerides. Indeed phospholipids appear to pick up predominantly acids of the C_{16} and C_{18} series in various proportions, despite the fact that other fatty acids are available in the fatty acid pool. Thus there appear to be certain selective mechanisms operating in biological systems for the incorporation of fatty acids into phospholipids.

ACKNOWLEDGMENT

GLC analysis of castor seed phospholipid fatty acids by P. K. Rajy, Texas A & M College, College Station, Texas.

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[Received Sept. 20, 1966]

The Effect of Antioxidant Deficiency on Tissue Lipid Composition in the Rat. I. Gastrocnemius and Quadriceps Muscle

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ABSTRACT

The gastrocnemius and quadriceps muscle phospholipids of the antioxidant-deficient rat fed a source of both linoleate and linolenate showed a progressive net increase in arachidonate, a progressive net decrease in all other polyunsaturated fatty acids, and there was a concomitant accumulation of fluorescent pigment of the lipofuscin or ceroid type in the tissue. An increased incorporation of intraperitoneally injected, isotopically labeled acetate into not only arachidonate but also the other higher polyunsaturated fatty acids, was observed. The net loss of the higher polyunsaturated fatty acids from the membrane lipids (presumably via lipid peroxidation) apparently was partially compensated by a homeostatic mechanism which involved conversion of the available precursors, linoleate and linolenate, to the higher polyunsaturated fatty acids. The rates of decrease of the polyunsaturated fatty acids in the muscle phospholipids and accumulation of fluorescent pigment in the tissue were correlated with the rate of production of creatinuria.

INTRODUCTION

THE NUTRITIONAL ASPECTS of the production of creatinuria in the tocopherol-deficient rat as a sign of the onset of nutritional muscular dystrophy have been explored in detail. Tissue lipid fatty acid composition has been correlated with dietary fatty acid composition (1,2). The animals' requirement for tocopherol as a lipid antioxidant[1] has been subjected to kinetic analysis (3,4). The polyunsaturated fatty acid (PUFA) content of the tissue lipids has been shown to be altered in the antioxidant-deficient animal (5-7) and this phenomenon appears to be related to the formation of lipofuscin or ceroid pigment (8-9).

Certain nonlipid factors, sulfur amino acids and biologically available selenium, have some effect on the lipid antioxidant-PUFA relationship as demonstrated by growth response and delay in the onset of creatinuria (1-4). The present report seeks to correlate the changes in tissue lipid composition in the antioxidant-deficient animal with previous nutritional studies and thus permits investigation of the mode of action of selenium and methionine at the cellular or subcellular level. Consideration has also been given, of necessity, to the homeostatic mechanisms resisting alteration in tissue lipid composition.

EXPERIMENTAL

Male, weanling rats of the Sprague-Dawley strain were fed a series of diets which duplicated those used in previous nutritional studies and which are described in detail elsewhere (1,2). The basal ration contained 0.04 ppm of selenium as determined by neutron activation analysis.[2] The division of animals into groups and the dietary protocol is described in Table I. Control groups received 15 mg *d*-α-tocopheryl acetate/kg rat body weight/week divided into three equal doses, dissolved in coconut oil, and administered by dropper directly to the animals. Previous experience (1,2) has demonstrated that such animals may be described as adequately tocopherol-supplemented in terms of prevention of creatinuria. Antioxidant status of the animals was assessed by periodically determining the excretion of creatine and creatinine in 24-hr urine samples by the method of Bonsnes and Taussky(11).

Animals were sacrificed periodically and liver, muscle, and in some cases testes were rapidly excised for histological examination by ultraviolet light-fluorescent microscopy (12) and for lipid analysis. Lipids were extracted

[1] The term "antioxidant" as used in this series of papers refers to any and all nontoxic, fat-soluble lipid antioxidants capable of being absorbed and retained to a significant degree by cellular lipids. Although *d*-α-tocopherol is the "normal" lipid antioxidant, all other lipid antioxidants which meet these criteria have been reported to be capable of replacing α-tocopherol (10). This does not imply that *d*-α-tocopherol has no other biological function, or that any of the synthetic antioxidants are as effective in terms of nontoxicity or biological distribution and storage at the subcellular level as *d*-α-tocopherol. Rather, the general term "antioxidant" is used to describe the mode of action of the compounds specifically under investigation.

[2] Activation Analysis Service, General Atomic Division of General Dynamics, San Diego, Calif.

TABLE I
Division of Animals into Groups

Dietary fat (%)	Supplementation[a] Selenium (ppm)	Methionine (%)	Tocopherol (mg/kg/wk)	No. of animals
"Trienoic" (12.5%)	0	0	0	40
	0	0	15	50
"Trienoic"[b] (7.5%)	0.13	0.4	0	66
	0.13	0.4	15	66
	0.13	0.4	DPPD[c]	68
"Trienoic" (7.5%)	0	0.4	0	20
	0	0.4	1.2	20
	0	0.4	15	10
	0	0	1.2	20
	0	0	15	10
	0.13	0.4	0.4	20
	0.13	0.4	15	10
"Monoenoic" (7.5%)	0	0	0	20
	0	0	0.6	20
	0	0	15	10
	0.13	0.4	0	20
	0.13	0.4	0.4	10
	0.13	0.4	15	10

[a] The basal ration contained casein (21.8% of calories), dextrose, salts 446 and vitamins as described previously (1,2), and 0.04 ppm of selenium as determined by neutron activation analysis. (Activation Analysis Service, General Atomic Division, General Dynamics, San Diego, Calif.)

[b] After 3 weeks the supplementation with selenium and methionine was discontinued for one-half of the rats in each of these 3 groups.

[c] 45 mg N,N′-diphenyl-para-phenylenediamine (DPPD)/kg rat body weight/week.

into methylal-methanol (4:1 v/v) containing 0.01% α-tocopherol as described previously (13) and care was taken to include this same antioxidant in the solvents used at each stage of the subsequent processing of the lipid. Phospholipid phosphorus was determined by the method of Zilversmit and Davis (14). Neutral lipids and phospholipids were separated by thin-layer chromatography (TLC) (15). After transmethylation, fatty acid composition was determined by gas-liquid chromatography (GLC) as described previously (13).

In one experiment approximately 0.2 μc/g rat of 1-C^{14}-acetate (specific activity 2.0 μc/μmole) was injected intraperitoneally 12 hr prior to sacrifice. To facilitate the study of the higher PUFA, usually present in rather small quantities in tissue, it was necessary to feed a dietary fat which would depress the tissue levels of essential fatty acids, specifically arachidonate, and contribute a source, linolenate, of the higher nonessential PUFA. For this specific reason the so-called "trienoic" fat, (Table I), was fed in this experiment. Muscle phospholipid fatty acid methyl esters were separated by degree of unsaturation (16) using 750 μ layers of Silica Gel G containing 20% silver nitrate (w/w) developed in ethyl ether-petroleum ether (60:40 v/v). Usually the muscle phospholipid fatty acids from two animals were pooled and separated on a single 200×200 mm plate. Bands were located by spraying with fluorescein and viewing under ultraviolet light. Eluted fractions were tested for identity and purity by GLC. Ester content was determined by the hydroxamic acid method of Clayton et al. (17), and appropriate aliquots were assayed for radioactivity in a liquid scintillation counter.

RESULTS

Creatine and creatinine excretion of control, adequately antioxidant supplemented animals, was maintained at "normal levels" consistent with previous experiments and the various experimental groups became "deficient" by the criterion of significant creatinuria after approximately the expected periods of tocopherol depletion or of maintenance on inadequate levels of antioxidant (1,2). Similarly, the gastrocnemius and quadriceps muscle lipid fatty acid composition of control, adequately antioxidant supplemented rats, was in excellent agreement with previously published data (1, 2). Analyses of liver and testicular lipids will be reported elsewhere (18,19).

The percentages of at least 23 fatty acids and two fatty aldehydes were computed for each of about 400 muscle phospholipid samples. Sequential sacrifices disclosed a general progressive decrease in all PUFA except arachidonate when antioxidant-deficient animals were compared to the appropriate controls. A progressive net increase in the arachidonate content of the muscle phospholipids was observed in the antioxidant-deficient animals. The magnitude of the fatty acid changes appeared to be related to the relative rapidity of the production of creatinuria (Table II). Certain aspects of the interrelations existing between the rates of decrease in concentration of the various individual PUFA in terms of their degree of unsaturation, the kinetics of in vivo lipid peroxidation and the rate of production of the antioxidant-deficiency sign, creatinuria, will be considered in detail elsewhere (20).

Low Selenium—Low Methionine Diets

When the so-called trienoic fat (62.2% saturated, 8.2% monoenoic, 5.9% dienoic, and 23.7% trienoic fatty acids) was fed in an antioxidant-deficient basal diet suboptimal in sulfur amino acids and biologically available selenium, correlation coefficients (21) indicated

TABLE II
Alteration of Muscle Phospholipid Fatty Acid Composition

Dietary fat	Supplementation: Selenium (ppm)	Methionine (%)	Tocopherol (mg/kg/wk)	Time (weeks)	Phospholipid fatty acids (% of control level)[a]: 22:6	22:5	20:4	No. of animals	Onset of creatinuria (weeks)
"Trienoic"	0	0	0	25	31	75	179	31	3
	0	0.4	0	20	34	78	131	17	3
	0.13	0.4	0	25	69	73	118	20	7
	0	0	1.2	30-38	69	84	108	6	12
	0	0.4	1.2	30-41	84	91	118	8	13
	0.13	0.4	0.4	35-41	76	82	108	5	12
"Monoenoic"	0	0	0	35-41	83	—[b]	116	5	10
	0	0	0.6	38-41	104	—	99	4	31
	0.13	0.4	0	30-38	92	—	104	6	22
	0.13	0.4	0	41-44	100	—	101	5	22

[a] Number of animals in control groups as stated in Table I.

[b] The tissue level of this fatty acid was negligible in rats fed the "monoenoic" fat.

that significant linear relationships were obtained for the logarithms of the percentages of linoleate, docosahexaenoate, and arachidonnate in the muscle phospholipid fatty acids versus the time of tocopherol depletion ($p<0.001$, $p<0.02$ and $p<0.001$, respectively). Compared to the appropriate adequately tocopherol-supplemented rats the average percentages of linoleic, docosapentaenoic, and docosahexaenoic acids were significantly lower ($p<0.001$) and the average percentage of arachidonic acid was significantly higher ($p<0.001$). The data have been presented in detail in Fig. 1 A, B, C, D with curves fitted

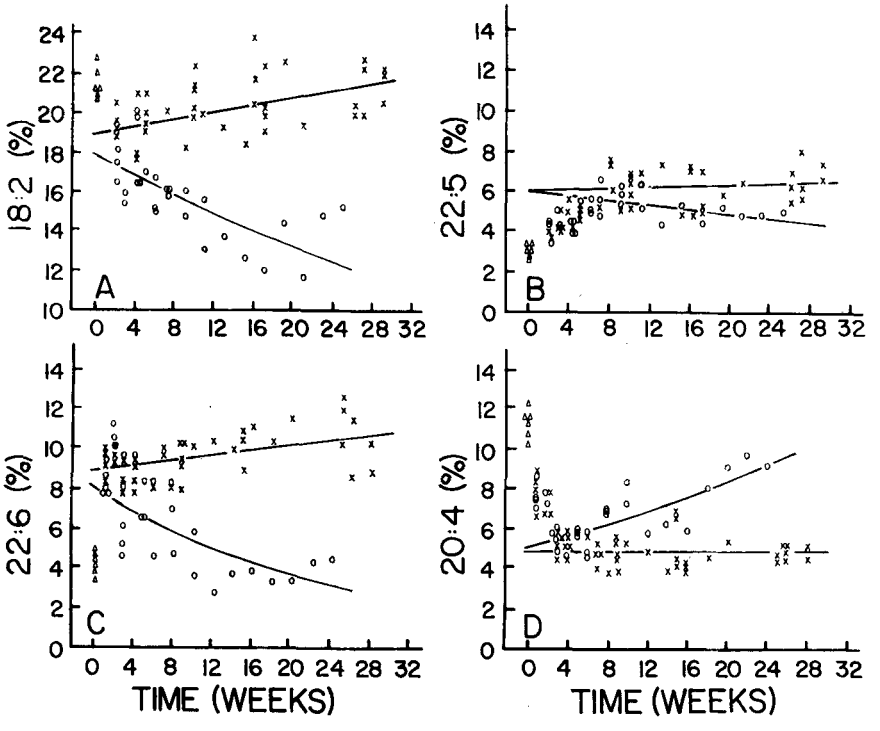


FIG. 1. Percentage of A) linoleic acid; B) docosapentaenoic acid; C) docosahexaenoic acid; and D) arachidonic acid in the phospholipid fatty acids of gastrocnemius and quadriceps muscles of tocopherol-deficient (O) and tocopherol supplemented (X) rats fed the "trienoic" fat at the 12.5% level in the basal ration. Triangles (Δ) denote weanlings as received at the start of the experiment. Lines have been fitted to the data by the method of least squares (21).

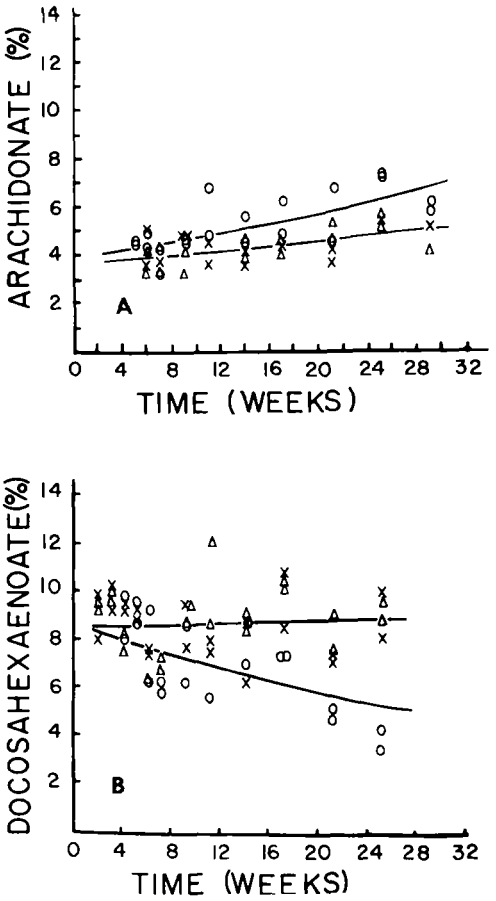


FIG. 2. Percentages of *A*) arachidonic acid and B) docosahexaenoic acid in the phospholipid fatty acids of the gastrocnemius and quadriceps muscles of tocopherol-deficient (O), tocopherol supplemented (X), and N,N'-diphenyl-paraphenylenediamine supplemented (Δ) rats fed the "trienoic" fat at the 7.5% level in the basal ration supplemented with 0.4% *dl*-methionine and 0.13 ppm of selenium as sodium selenite. Lines have been fitted to the experimental data by the method of least squares (21).

to the experimental points by the method of least squares (21). A small, but significant ($p<0.001$), decrease in phospholipid linolenate in the antioxidant-deficient group compared to the antioxidant-supplemented group, $1.5\% \pm 0.11\%$ versus $2.4\% \pm 0.09\%$, was also noted but not graphed. Small, but significant, decreases in neutral lipid linoleate and linolenate were also observed in this group in comparison to the controls. The values were $5.1\% \pm 0.21\%$ versus $5.8\% \pm 0.18\%$ ($0.02 > p > 0.01$) for linoleate and $5.8 \pm 0.22\%$ versus $6.8 \pm 0.32\%$ ($p=0.05$) for linolenate.

The so-called monoenoic fat (11.7% saturated, 81.1% monoenoic, and 7.2% dienoic acids) had the same total unsaturation (iodine value 82 [22]) as the trienoic fat, but production of creatinuria required 10 weeks. After a protracted period, 35-41 weeks, docosahexaenoate decreased to 83% and arachidonate increased to 116% of the control levels (Table II).

Selenium and Methionine Supplemented Diets

Addition of both selenium, 0.13 ppm as sodium selenite, and supplemental sulfur amino acid, 0.4% *dl*-methionine, to the antioxidant-deficient diet containing the trienoic fat had the expected (1,2) effect on the rate of production of creatinuria (Table II), and approximately twice as long an experimental period was required for the production of this specific sign of antioxidant deficiency. The increase in muscle phospholipid arachidonate also proceeded more slowly (Fig. 2,A), as did the decrease in the other PUFA. Of the other PUFA, only the data for docosahexaenoate have been presented in detail (Fig. 2,B). Note that the slope of this curve is approximately one-half the slope found in Fig. 1,C. In this particular experiment *d*-α-tocopheryl acetate was administered by dropper, to one-half of the adequately antioxidant-supplemented control rats while the others received the synthetic antioxidant N,N'-diphenyl-p-phenylenediamine, DPPD.

When the monoenoic fat was fed in this diet, production of creatinuria required 22 weeks and alteration of the muscle phospholipid fatty acid pattern was not evident after 41-44 weeks (Table II).

Methionine Supplemented Diet

Sixteen rats fed the trienoic fat in the basal ration supplemented with 0.4% *dl*-methionine were sacrificed between the 12th and 27th weeks of the experiment. The regression curve for docosahexaenoate had a slope very similar to that obtained for the group fed the unsupplemented antioxidant-deficient basal ration, (Fig. 1). In muscle phospholipids of the methionine supplemented group, docoshexaenoate decreased to one-half the level seen in the appropriate control group in 13 weeks (slope ± 3 standard deviations yields values of 12.8 and 13.1 weeks) while the comparable period for the group fed the unsupplemented basal ration was 15 weeks (slope ± 3 standard deviations yields values of 15.0 and 15.2 weeks).

The average percentage of arachidonate of this group was 1.3 times the level seen in the appropriate adequately antioxidant-supplemented control group and this difference was statistically significant ($p<0.001$), as shown in Table II.

Effect of Inadequate Tocopherol Supplementation

Animals fed the trienoic fat containing basal ration alone, with added selenium and methionine, or with only added methionine were administered levels of *d*-α-tocopheryl acetate such that all three groups developed significant creatinuria after approximately the same time, 12-13 weeks (Table II). The significant ($p<0.01$) decrease in docosahexaenoate in the group receiving supplemental selenium and methionine corresponds to an extrapolated decrease to one-half the level seen in the appropriate adequately antioxidant-supplemented controls in 63 weeks. In all three cases docosahexaenoate decreased and arachidonate increased in the muscle phospholipid fatty acids of the antioxidant-deficient after a prolonged experimental period.

Muscle Phospholipid Levels

A small, gradual decrease in the phospholipid level of the gastrocnemius and quadriceps muscles was noted in control, adequately antioxidant-supplemented rats fed the trienoic fat (Table III). Comparison of tissue phospholipid levels must therefore take into consideration the time of sacrifice. The changes in phospholipid fatty acid composition described above are apparently representative of net changes in tissue levels of the various fatty acids.

Fluorescent Pigment

The relative quantity of fluorescent pigment seen in unstained muscle sections by ultraviolet light microscopy was approximated on a 0 to 4+ scale (Table IV). Ceroid or lipofuscin type pigments appeared to accumulate most rapidly in the tissue of the groups showing the greatest decreases in phospholipid PUFA.

Interconversion of Phospholipid PUFA

Administration of 1-C^{14}-acetate resulted in incorporation of the radioactive isotope into all of the higher PUFA. All data were obtained from the groups fed the trienoic fat in the basal ration containing supplemental selenium and methionine.

Thin-layer chromatography on silver nitrate-containing silica gel layers was noted to separate PUFA not only by degree of unsaturation but also by fatty acid chain length and

TABLE III

Gastrocnemius and Quadriceps Muscle Phospholipid Levels

Dietary fat	Supplementation: Selenium (ppm)	Supplementation: Methionine (%)	Supplementation: Tocopherol (mg/kg/wk)	Phospholipid (mg/g tissue)	Average time of sacrifice (weeks)	No. of animals
"Trienoic"	0.13	0.4	0	8.7 ± 0.5[a]	10	20
	0.13	0.4	15	8.9 ± 0.6	10	18
	0.13	0.4	DPPD[b]	9.1 ± 0.5	10	17
	0.13	0.4	0.4	8.7 ± 0.3	27	11
	0.13	0.4	15	8.6 ± 0.5	28	9
	0	0.4	0	8.9 ± 0.4	19	16
	0	0.4	1.2	7.6 ± 0.6	35	8
	0	0.4	15	8.9 ± 0.9	17	7
	0	0	0	—	—	—
	0	0	1.2	8.0 ± 0.4	32	10
	0	0	15	8.4 ± 0.4	30	4
"Trienoic"[c]	0.13	0.4	0	9.2 ± 0.2	23	21
	0.13	0.4	15	9.3 ± 0.3	22	21
	0.13	0.4	DPPD	9.1 ± 0.4	22	20
"Monoenoic"	0	0	0	8.4 ± 0.7	30	14
	0	0	0.6	8.5 ± 0.3	35	9
	0	0	15	8.3 ± 0.6	29	8
	0.13	0.4	0	9.0 ± 0.6	34	11
	0.13	0.4	0.4	9.0 ± 0.5	37	9
	0.13	0.4	15	8.8 ± 1.0	35	6

[a] Average ± SEM

[b] N,N′-diphenyl-*p*-phenylenediamine 45.0 mg/kg rat/wk.

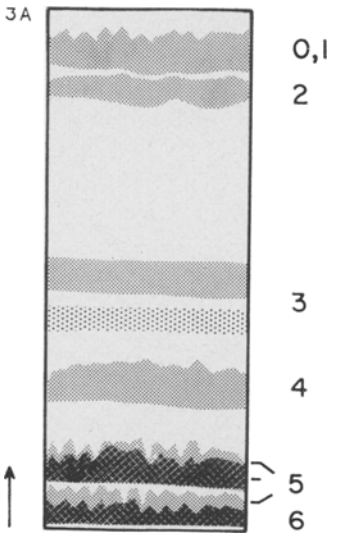
[c] This group received the supplemented selenium and methionine for only the first 3 weeks of the experiment and is not included elsewhere in this report. Data furnished for comparison only.

TABLE IV
Appearance of Fluorescent Pigment in the Muscle at Various Time Periods (Weeks)

Dietary fat	Supplementation: Selenium (ppm)	Supplementation: Methionine (%)	Supplementation: Tocopherol (mg/kg/wk)	Weeks	Average pigment score[a]	No. of animals
"Trienoic"	0	0	0	12	1.0	3
				16	2.0	3
				20	3.0	3
	0	0.4	0	14	0.7	3
				17	3.3	3
	0.13	0.4	0	15	0.3	3
				19	1.0	3
				23	2.0	3
				25	3.3	3
	0	0	1.2	35-41	0	5
	0	0.4	1.2	35-38	0	5
	0.13	0.4	0.4	35-38	0.7	3
"Monoenoic"	0	0	0	35-41	0	6
	0.13	0.4	0	35-38	0.3	6

[a] Individuals graded on a 0-4+ scale.

location of the double bonds. Photographs of the plates have been included (Fig. 3,A and B) to illustrate this previously unreported phenomenon. Separation of the fatty acid methyl esters by silver nitrate-TLC using ethyl ether-petroleum ether (60:40 v/v) as the developing solvent is illustrated in Fig. 3,A. Saturated and monoenoic esters are not resolved in this solvent system. The hexaenoate fraction obtained from these plates was usually contaminated with pentaenoate. In a few cases another solvent system, chloroform-methanol 7:1 (v/v), was used (Fig. 3,B) to obtain a pure hexaenoate fraction. Note the presence of 2 triene and 3 pentaene bands in Fig. 3,A. These fine separations were observed but not utilized in the present study since the degree of tailing encountered would have required rechromatographing these highly oxygen-labile materials several times to obtain suitable and analytical samples.



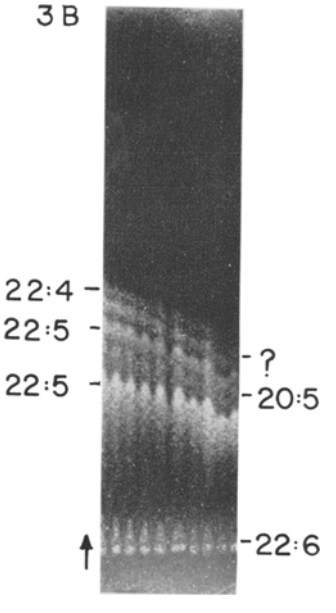


FIG. 3. Separation of rat muscle phospholipid fatty acid methyl esters by degree of unsaturation. Silica Gel G-$AgNO_3$ (3-1 w/w) 750μ thick developed with A) diethyl ether-petroleum ether (60:40 v/v). Separation of the higher polyunsaturated fatty acids with B) chloroform-methanol (7:1 v/v). Sprayed with fluorescein photographed under ultraviolet light. In A) numbers in the margin refer to the number of double bonds in the fatty acids. This figure is a tracing of the actual photograph. In B) the numbers refer to fatty acid chain length and number of double bonds.

TABLE V

Incorporation of 1-C^{14} Acetate into Muscle Phospholipid Fatty Acids in Tocopherol-Deficient and Tocopherol or DPPD-Supplemented Rats

	Muscle phospholipid fatty acids								
	Trienoic			Tetraenoic			Penta- and hexaenoic[a]		
	Deficient	Supplemented	Ratio deficient to supplemented	Deficient	Supplemented	Ratio deficient to supplemented	Deficient	Supplemented	Ratio deficient to supplemented
Weeks	μμc/g[b] muscle	μμc/g muscle		μμc/g muscle	μμc/g muscle		μμc/g muscle	μμc/g muscle	
3	854	750	1.1	81	75	1.1	371	415	0.9
4	1627	1795	0.9	602	102	5.9	307	391	0.8
5	183	99	1.8	—	—	—	74	126	0.6
7	640	525	1.2	—	22	—	426	310	1.4
9	648	270	3.8	32	22	1.4	209	165	1.3
11	—	191	—	211	40	5.3	184	238	0.8
14	1028	318	4.7	122	80	1.5	350	168	2.1
17	109	69	1.6	23	20	1.1	166	118	1.4
21	200	201	1.0	70	59	1.2	180	132	1.4
29	184	43	4.3	173	117	1.5	233	160	1.5

[a] Separation of pentaenoic acids from hexaenoic acid (Ag NO_3-TLC 7:1 chloroform-methanol) in a few experiments suggested that the excess incorporation of radioactivity into this fraction was almost completely into the pentaenoic acids.

[b] Incorporation based on injection of 0.2 μc/g rat of 1-C^{14}-acetate (specific activity 2.0 mc/mmole).

After 12 hr, larger net quantities of the isotopically labeled acetate, per gram of tissue, were incorporated into the PUFA of the antioxidant-deficient animals than into the PUFA of the *d*-α-tocopheryl acetate or DPPD supplemented rats (Table V). This increased tetraenoic acid synthesis, largely arachidonate, is in agreement with the data of Bernhard et al. (6) and is consistent with the net increase in arachidonate observed in the tissue (Fig. 1,C). The data for the higher polyenoic acids suggests a generalized increase in the processes of fatty acid chain elongation and desaturation occurring in both the essential (ω 6) and nonessential (ω 3) series of PUFA rather than a simple and specific increase in arachidonate formation.

DISCUSSION

The effect of various types of dietary fats, high in linoleate, low in linoleate, high in linolenate, and high in higher, nonessential PUFA, on nutritional muscular dystrophy were considered in detail in previous reports (1-4). Decreases in muscle phospholipid PUFA were found to occur in tocopherol-deficient rats fed all of these types of dietary fats (1). Choice of a dietary fat for use in the present study, however, was restricted by the desire to demonstrate and clarify in muscle the net increase in arachidonate noted in the livers of tocopherol-deficient rats by Bernhard et al. (6) and by Harmon, Witting and Horwitt (23).

In the usual diet containing linoleate as the sole PUFA, only small amounts of nonessential PUFA are present in the muscle phospholipids. Using such diets it would, therefore, be quite difficult to resolve the peroxidative decrease in arachidonate, the most highly unsaturated PUFA present in any quantity in the tissue, from the increased synthesis of arachidonate. Inclusion of linolenate in the diet produced phospholipids containing 20% of the more highly unsaturated nonessential PUFA and reduced arachidonate from 20% to 5% of the fatty acids. Since the pentaenoic and hexaenoic acids are 1.5 to 2 times as susceptible to peroxidation in vivo (1-4) as arachidonic acid, it was possible to distinguish, partially, the decrease in the PUFA attributed to lipid peroxidation from the increased synthesis of arachidonate. In addition it was possible to demonstrate that the net increase in arachidonate was part of a more general phenomenon since an increased incorporation of isotopically labeled acetate into the higher nonessential PUFA was also observed.

The more highly unsaturated PUFA would seem to be necessary as constituents of membrane lipids. In essential fatty acid-deficiency, oleate and palmitoleate are converted to trienoic acids (24). In an antioxidant deficiency where the more unsaturated PUFA are removed from the system, presumably via lipid peroxidation, the increased conversion of the available precursors, linoleate and linolenate, to the higher PUFA would seem to reflect a homeostatic process attempting to maintain a

"normal" membrane lipid fatty acid composition.

Direct evidence is not available to relate the decrease in muscle phospholipid PUFA to the actual occurrence of lipid peroxidation in vivo. The slopes of the curves describing the decrease in docosahexaenoate content of the muscle phospholipids in the various groups are in the same relation to one another as are the rates of production of creatinuria in these same groups (Table II and Fig. 1,C and 2,B). A detailed analysis of the changes in fatty acid composition of the muscle phospholipid will be presented in a subsequent paper (20).

Hartroft (25) has shown that prior to the accumulation of fluorescent pigment there is an accumulation of pre- or pro-ceroid pigments, soluble in the dehydrating solvents employed in the usual histological procedures. Determination of these earlier pigments might yield a closer time relation between decrease in phospholipid PUFA and accumulation of a material thought (26-29) to be a combination of oxidized lipid and protein.

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The Effect of Antioxidant Deficiency on Tissue Lipid Composition in the Rat. II. Liver

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ABSTRACT

The hepatic phospholipids of the antioxidant-deficient rat fed a source of both linoleate and linolenate showed a progressive net decrease in eicosapentaenoate, a progressive net increase in arachidonate, and there was a concomitant accumulation of fluorescent pigment of the lipofuscin or ceroid type in the tissue. An increased incorporation of isotopically labeled acetate into both the tetraenoic and pentaplus hexaenoic acid fractions was also noted, indicating that the disappearance of polyunsaturated fatty acids was partially countered by increased synthesis. Comparable results were obtained on diets containing either suboptimum or adequate levels of biologically available selenium. Vesicular dilation of the endoplasmic reticulum was noted in animals fed the tocopherol-deficient diet. In separate experiments using a necrogenic diet containing torula yeast, these subcellular alterations were found to be prevented by tocopherol but not by selenium, although selenium supplementation did prevent macroscopically observable damage.

INTRODUCTION

ON THE BASIS of electron microscopic studies, Sulkin and Sulkin (1) reported that the liver was adversely affected in rats fed a tocopherol-deficient diet for prolonged periods. The damage was much milder than the massive liver necrosis which is rapidly produced on diets containing torula yeast (2). Bernhard et al. (3,4) have recently described an increased incorporation of isotopically labeled acetate into arachidonate and a net increase in the arachidonate content of the liver of antioxidant-deficient rats.[2] A transient increase in liver arachidonate was observed by Harmon, Witting and Horwitt (5) under conditions of simultaneous tocopherol and essential fatty acid depletion in rats fed a casein diet relatively low in biologically available selenium. Using a diet stated to be adequate in selenium, however, Bieri and Andrews (6) were unable to detect a rise in liver arachidonate.

Studies on gastrocnemius and quadriceps muscle phospholipid fatty acids (7) and testicular phospholipid fatty acids (8) have suggested that the rise in arachidonate originally noted in rat liver may be only part of a more complex, general homeostatic process occurring in all tissues adversely affected by antioxidant-deficiency. In the present investigation an effort was made to determine the effects of dietary fat, selenium, sulfur amino acids, and low levels of α-tocopherol on the composition of the liver lipids and on the biosynthesis of the higher polyunsaturated fatty acids.

EXPERIMENTAL

The animals used in the present study were among those described in a previous paper relating to muscle phospholipid fatty acid composition (7).

While this study was in progress, tissue was available for electron microscopy from rats fed the basal, torula yeast, necrogenic diet of Schwartz (2), the basal diet, plus 800 μg of *d*-α-tocopheryl acetate per 100 g diet, and the basal diet plus 0.36 mg sodium selenite per 100 g diet. According to Schwartz and Mertz (9) the levels of supplemental tocopherol and selenium described above should protect all the rats against liver necrosis. The tissue was fixed as described by Millonig (10) and dehydrated and blocked according to the method of Luft (11). Sections stained with lead hydroxide (12) were examined in a Hitachi HS-7 electron microscope.

RESULTS

The first group of rats were fed an antioxidant-deficient basal ration (13) with 12.5% synthetic "trienoic" fat (62.2% saturated, 8.2% monoenoic, 5.9% dienoic and 23.7% trienoic fatty acids) containing 0.04 ppm of

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[2] See *footnote 1*; Witting, L. A., and M. K. Horwitt. Lipids *2*, 89-96 (1967).

TABLE I

Effect of Tocopherol-Deficiency on the Fatty Acid Composition of Rat Liver Phospholipids and the Accumulation of Fluorescent Pigment

Group	Weeks on experiment	Arachidonate (%)	Weeks on experiment	Eicosapentaenoate (%)	Weeks on experiment	Average fluorescent pigment[a]
Supplemented[b]	11-33	6.7 ± 0.3 (37)[c]	11-33	7.4 ± 0.3 (37)	11-33	0 (37)
Deficient	11-23	6.6 ± 0.5 (11)	15-19	7.6 ± 0.3 (6)	12	0 (3)
	27-30	8.1 ± 0.8 (7)[d]	23-20	5.2 ± 0.6 (13)[e]	14	1.3 (3)
					16	2.0 (3)

[a] Scored on a 0 to 4 scale.

[b] Half of group received 15 mg *d*-α-tocopheryl acetate and half received 45 mg N,N′-diphenyl-para-phenylene-diamine/kg rat body weight/week.

[c] Average ± SEM; number of animals in parentheses. All fatty acid percentages are expressed in terms of area of gas-liquid chromatographic elution diagrams.

[d] $p < 0.05$

[e] $p < 0.001$

selenium, as determined by neutron activation analysis,[3] largely present in the casein. For the first three weeks of the experiment, but not thereafter, this basal ration was supplemented with 0.13 ppm of selenium as sodium selenite and 0.4% *dl*-methionine. One-third of the group received 15 mg of *d*-α-tocopheryl acetate per kg rat body weight per week administered orally by dropper three times a week; another third similarly received 45 mg N,N′-diphenyl-paraphenylenediamine (DPPD). This diet supplied 1.5% of calories as linoleate.

During the first portion of the experimental period, 11-23 weeks, the liver phospholipids of the unsupplemented group contained the same level of arachidonate (Table I) as the tocopherol-supplemented, and DPPD-supplemented rats. Thereafter, 27-30 weeks, a significant ($p<0.05$) increase in arachidonate was noted in the tocopherol-deficient group. Similarly, up to 19 weeks the eicosapentaenoate level in the liver phospholipids of the unsupplemented group did not differ from that seen in the antioxidant-supplemented group (Table I). Thereafter, 23-30 weeks, a significant ($p<0.001$) decrease in eicosapentaenoate levels was noted in the antioxidant-deficient group. Eicosapentaenoate is the major PUFA

[3] Activation Analysis Service, General Atomic Division of General Dynamics, San Diego, California.

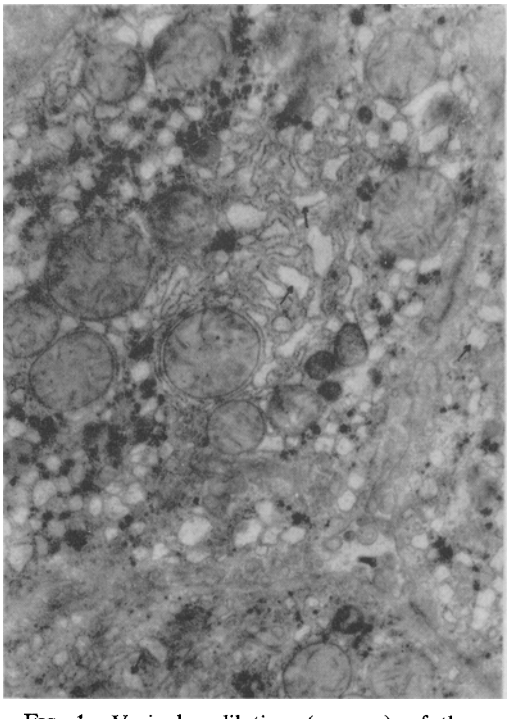

FIG. 1. Vesicular dilation (arrows) of the endoplasmic reticulum in the liver of a rat fed a tocopherol-deficient, casein diet, containing the "trienoic" fat. The mitochondria show no obvious morphological changes (Mag. × 12,000).

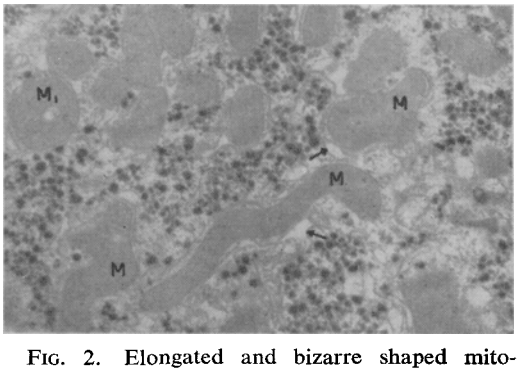

FIG. 2. Elongated and bizarre shaped mitochondria (M) in the liver of rats fed the necrogenic, torula yeast diet (1). Vesicular dilation of the endoplasmic reticulum is also noted (arrows) (Mag. × 13,000).

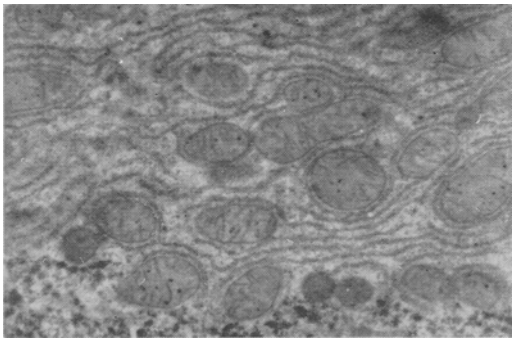

FIG. 3. Appearance of the liver cells in a rat fed the necrogenic torula yeast diet supplemented with tocopherol. The endoplasmic reticulum and mitochondria are essentially normal in appearance (Mag. × 19,000).

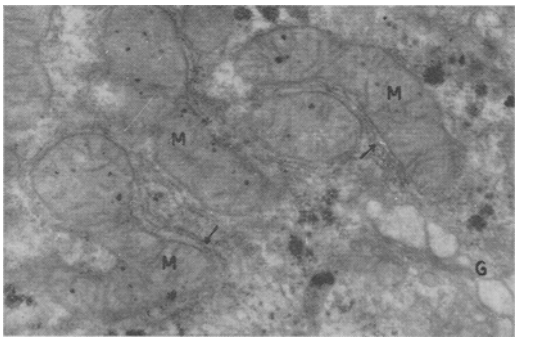

FIG. 4. Appearance of the liver cells in a rat fed the necrogenic, torula yeast diet supplemented with sodium selenite. Elongated and bizarre shaped mitochondria (M) are present. The golgi zone (G) is very prominent and the endoplasmic reticulum shows a tendency to surround individual mitochondria (arrows) (Mag. × 22,000).

of the hepatic phospholipids when the "trienoic" fat is fed.

The alterations in phospholipid fatty acid composition were not associated with macroscopic evidence of hepatic damage. During the latter portion of the experiment (approximately 20-26 weeks), vesicular dilation of the endoplasmic reticulum was readily demonstrated by electron microscopy (Fig. 1). These changes in the endoplasmic reticulum were noted, after four weeks, in animals fed the basal necrogenic diet containing torula yeast (2). In addition, in the latter case, bizarre shaped mitochondria were evident (Fig. 2). The subcellular changes noted in this electron micrograph appear to correspond to those reported as occurring prior to gross massive liver necrosis by Piccardo and Schwartz (14). Supplementation of the necrogenic diet with tocopherol (Fig. 3) resulted in essentially normal appearing endoplasmic reticulum and mitochondria, whereas supplementation with biologically available selenium did not (Fig. 4).

In another experiment this same "trienoic" fat was fed at the 7.5% level in each of three antioxidant-deficient diets. The first was the basal ration, the second was supplemented with both selenium (0.13 ppm) and methionine (0.4% *dl*), and the third was supplemented with only methionine (0.4% *dl*). The levels of antioxidant supplementation are noted in Table II. After the designated experimental periods, significant increases in liver phospholipid arachidonate were observed (Table II) in three groups. Small, nonsignificant changes in eicosapentaenoate levels were noted, but

TABLE II

Effect of Dietary Fat Composition and Supplementation with Selenium, Methionine and Low Levels of α-Tocopherol on Liver Phospholipid Fatty Acid Composition

Dietary fat	Supplements: Selenium (ppm)	Methionine (%)	Tocopherol (mg/kg/wk)	Experimental period (weeks)	No. of samples	Phospholipid arachidonate (%)	Significance
"Trienoic"	0	0	15	22-38	4	5.2	
	0	0.4	15	18-38	8	5.5	
	0.13	0.4	15	12-22	7	5.4	
Summation of adequately tocopherol supplemented rats				12-38	19	5.4 ± 0.3[a]	
"Trienoic"	0	0.4	0	18-27	9	6.9 ± 0.4	$p < 0.005$
	0	0	1.2	35-41	5	6.7 ± 1.0	$p < 0.05$
	0	0.4	1.2	35-41	4	6.4 ± 1.0	
	0.13	0.4	0.4	30-35	5	7.6 ± 0.3	$p < 0.001$
"Monoenoic"	0	0	15	22-38	5	8.0 ± 0.9	
	0.13	0.4	15	30-41	5	7.8 ± 0.9	
	0	0	0	30-38	7	7.6 ± 0.4	
	0	0	0.6	30-41	7	7.8 ± 0.6	
	0.13	0.4	0	30-44	11	7.8 ± 0.5	

[a] Average ± SEM

TABLE III

Effect of Dietary Fat Composition and Supplementation with Selenium, Methionine, and Low Levels of α-Tocopherol on Liver Phospholipid Level

Dietary fat	Supplements: Selenium (ppm)	Supplements: Methionine (%)	Supplements: Tocopherol (mg/kg/wk)	Experimental period (weeks)	No. of samples	Liver phospholipid (mg/g liver)
"Trienoic"	0	0	15	22-38	4	27.1
	0	0.4	15	18-38	8	27.0
	0.13	0.4	15	12-22	7	27.2
	Summation of adequately tocopherol supplemented rats			12-38	19	27.1 ± 0.8[a]
	0	0.4	0	12-27	14	24.9 ± 0.5
	0	0	1.2	32-41	9	26.8 ± 0.9
	0	0.4	1.2	35-41	4	27.2 ± 0.9
	0.13	0.4	0.4	18-35	10	26.5 ± 1.0
"Monoenoic"	0	0	15	22-38	5	27.9 ± 2.2
	0.13	0.4	15	30-41	6	27.6 ± 1.2
	0	0	0	22-41	8	29.2 ± 0.9
	0	0	0.6	30-41	7	28.3 ± 1.1
	0.13	0.4	0	30-44	12	30.8 ± 0.5

[a] Average ± SEM

have been omitted from this table. While the "trienoic" fat supplied 1% of calories as linoleate, the hepatic arachidonate level was depressed by the dietary linolenate.

When a "monoenoic" fat (11.7% saturated, 81.1% monoenoic and 7.2% dienoic acids) of the same level of total unsaturation (iodine value 82) as the "trienoic" fat was fed in these same diets, liver arachidonate levels were not altered within the experimental period, 38-44 weeks (Table II). This diet supplied 1.2% of calories as linoleate.

Measurement of liver phospholipid by the method of Zilversmit and Davis (15) (Tables I and III) indicated that the increase in arachidonate and decrease in eicosapentaenoate in the phospholipid fatty acids were net changes in the liver content of these fatty acids. Fluorescent pigment of the lipofuscin or ceroid type (16) was noted to accumulate in the livers of the antioxidant-deficient rats (Table I).

In the first experiment, intraperitoneal injection of approximately 0.2 μc of 1-C^{14}-acetate (specific activity 2.0 μc/μmole) per gram rat, 12 hr prior to sacrifice resulted in labeling of the higher polyunsaturated fatty acids (8). The fatty acid methyl esters were separated by degree of unsaturation on 750 μ layers of Silica Gel G containing 20% silver nitrate (w/w) (17) using ethyl ether-petroleum ether, 60-40, (v/v) as the developing solvent. Samples containing 50-100 mg of methyl esters were normally applied to a single 20-cm × 20-cm plate. Identity and purity of fractions eluted with water saturated-ethyl ether was determined by gas-liquid chromatography. Ester content was determined by the hydroxamate method of Clayton et al. (18) and the radioactivity present in appropriate aliquots determined by use of a liquid scintillation counter.

The increased incorporation of labeled acetate into the tetraenoic fraction (Table IV) is consistent with the net increase of arachidonate actually observed. This fraction also contained a very small amount of a docosatetraenoic acid which could be isolated separately. While this fatty acid appeared to have the same specific activity as arachidonate, the

TABLE IV

Incorporation of 1-C^{14} Acetate into Hepatic Fatty Acids in Tocopherol-Deficient and Tocopherol or DPPD-Supplemented Rats

Hepatic Fatty Acid	Deficient (mμc/g liver)[b]	Supplemented[a] (mμc/g liver)	Ratio of Deficient to Supplemented	Significance
Trienoic	2.15 ± 0.4 (17)[c]	3.42 ± 0.5 (28)	0.6	$0.10 > p > 0.05$
Tetraenoic	3.33 ± 0.5 (16)	2.19 ± 0.3 (30)	1.5	$0.025 > p > 0.020$
Pentaenoic & Hexaenoic	7.68 ± 0.7 (14)	5.53 ± 0.6 (31)	1.4	$0.05 > p > 0.025$

[a] One-half of group received *d*-α-tocopheryl acetate (15 mg/kg rat/week) and one-half received N,N'-diphenyl-p-phenylenediamine (DPPD) (45 mg/kg rat/week).

[b] Incorporation based on the intraperitoneal injection of 0.2 μc of 1-C^{14} acetate (specific activity 2.0 μc/μM) per gram rat.

[c] Average ± SEM, number of samples in parentheses.

techniques employed in the present study were not of sufficient sensitivity to permit a definite statement in this regard.

Three pentaenoic acids and one hexaenoic acid were usually eluted as a single fraction. The docosapentaenoate with the essential fatty acid structure (22:5 ω 6)[4] was present in the liver lipids to the extent of only a few tenths of one per cent. In view of the extreme susceptibility of these highly unsaturated fatty acids to autoxidation, extensive purification was not undertaken. In a few cases the docosahexaenoate was isolated and incorporation of the isotope into this fatty acid in the liver appeared to be quite similar in the tocopherol-deficient and tocopherol or DPPD-supplemented rats. Note that an increased quantity of labeled acetate was incorporated into the pentaenoate plus hexaenoate fraction from the antioxidant-deficient group although the level of the major fatty acids in this fraction decreased in the liver. The data are stated in terms of actual incorporation of labeled acetate rather than specific radioactivity of the fatty acid fraction which would be affected by the alterations in fatty acid composition.

DISCUSSION

On the basis of electron microscopic studies of the liver, it would seem apparent that this tissue is adversely affected (vacuolization of the endoplasmic reticulum) in rats fed an antioxidant-deficient diet. These subcellular changes resemble those seen in the earliest stages of massive liver necrosis (14). In the present experiment, although complete protection was afforded by tocopherol, supplementation with only selenium did not suffice to prevent liver damage in animals fed the torula yeast-containing necrogenic diet. This would agree with the data of Hartroft (20). On this same diet he found that while either selenium or tocopherol prevented gross liver necrosis, only tocopherol prevented the accumulation of ceroid type pigment.

The hepatic phospholipids of the antioxidant-deficient rat appear to be altered in the same general manner as are the gastrocnemius and quadriceps muscle phospholipids. There is a progressive net decrease in the major, most highly polyunsaturated fatty acid in the tissue, eicosapentaenoate, a concomitant accumulation of fluorescent pigment of the lipofuscin or ceroid type, and a net increase in arachidonate. An increased incorporation of isotopically labeled acetate into both tetra- and penta-plus hexaenoic acids was also noted to occur. The stimulation of the enzymatic processes of fatty acid chain elongation and desaturation is therefore viewed as a homeostatic response to the "peroxidative destruction" of the higher PUFA which appear to be essential constituents of the membrane lipids. Net tissue lipid composition represents a balance between these competing processes.

The dietary fats used were low (1-1.5% of calories) but adequate in linoleate. Perhaps it is significant that the other two reports of increased hepatic arachidonate levels were based on animals transferred from high linoleate intakes to diets relatively low in linoleate content (3,5). Since the study of hepatic lipids was begun during, and was secondary to, an extended study of muscle lipids, a full representation of combinations and permutations of the non-lipid-variables was not available. Accordingly, the data are not as detailed as those presented for muscle (7) and testes (8). It is clear, however, that the "effect" of a fat-soluble lipid antioxidant-deficiency on the hepatic phospholipid fatty acid composition may be demonstrated on diets containing adequate levels of biologically available selenium and methionine.

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Technical assistance provided by Judith Krishnam, Ruth C. Nelson, M. Jane Morton, and V. N. Likhite.

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[4] The omega nomenclature of Rahm and Holman (19) which describes the position of the final double bond in the fatty acid molecule permits a simple description of the family relationships in the various fatty acid series, linoleate (ω 6), linolenate (ω 3), and oleate (ω 9).

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The Effect of Antioxidant Deficiency on Tissue Lipid Composition in the Rat. III. Testes

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ABSTRACT

Production of testicular degeneration in the antioxidant-deficient rat resembles encephalomalacia in the chick in its dependence on essential (ω6) fatty acids and is distinct from the generalized response to all polyunsaturated fatty acids seen in nutritional muscular dystrophy in the rat. The nonessential (ω3) polyunsaturated fatty acids, however, lower the essential fatty acid content of the testicular lipids only slightly, are not themselves incorporated into this tissue to any appreciable degree and thus do not show the inhibitory effect on production of the antioxidant-deficiency sign noted in the studies on encephalomalacia. A direct relationship between the essential fatty acid content of the testes and the rate of testicular degeneration was found, but no effects of biologically available selenium and sulfur amino acids were evident. As the liver and muscle, onset of antioxidant-deficiency is characterized by a decrease in the most highly unsaturated fatty acid in the tissue (22:5-ω6 in this case) and a net increase in arachidonate.

INTRODUCTION

THE BIOCHEMICAL changes occurring during testicular degeneration in the antioxidant-deficient rat are of particular interest for two distinct reasons. First, large quantities of an essential pentaenoic fatty acid (22:5ω6)[1] derived from linoleate (18:2ω6) via arachidonate (20:4ω6) are present in this tissue (2). Arachidonate is normally the highest essential polyunsaturated fatty acid (PUFA) present to any great extent in tissue lipids and it has been shown that there is a significant net increase in the arachidonate content of several tissues (3-6) of the antioxidant-deficient rat.[2] A study of the alterations in the docosapentaenoate content of the testes might provide information pertinent to the nature of this apparent deficiency response.

Second, nonessential PUFA (18:3ω3, 20:5ω3, 22:5ω3, 22:6ω3) are not readily incorporated into the lipids of the testes. The latter point suggests that the effect of manipulation of the fatty acid composition of the dietary fat on the course of development of an antioxidant-deficiency may differ from the effects noted when encephalomalacia in the chick (7-9) or nutritional muscular dystrophy in the rat (10,11) are studied.

EXPERIMENTAL

Tissue derived from antioxidant-deficient rats raised for other purposes (3,4) were utilized in the preliminary experiments. Except for the nature of the dietary fat and the levels of tocopherol supplementation, these rats were fed diets directly comparable to those described below.

Three hundred ninety-two male weanling rats of the Sprague-Dawley strain were fed a series of diets containing specially purified "tocopherol-free" fats of constant iodine value (12) 82.0. The so-called "dienoic" fat (saturated 43.5%, monoenoate 16.7%, dienoate 39.0%) was fed at the 1.2% and 7.5% levels to furnish 1.10% and 6.3% of calories as linoleate, respectively. A so-called "trienoic" fat (saturated 59.6%, monoenoate 9.9%, dienoate 7.3% and trienoate 23.2%) fed at the 7.5% level supplied 1.18% of calories as linoleate and 3.75% of calories as nonessential PUFA, linolenate, and a so-called "polyenoic" fat (saturated 57.8%, monoenoate 24.8%, dienoate 1.0%, trienoate 4.1%, tetraenoate 2.5%, pentaenoate 5.9% and hexaenoate 3.7%) also fed at the 7.5% level, supplied 0.16% of calories as linoleate and 2.73% of calories as nonessential PUFA.

The basal ration contained casein (21.8% of calories), dextrose, salts 446 (13), vitamins (14) and 0.04 ppm of selenium.[3] Supplemental quantities of selenium, 0.13 ppm as sodium

[1] The omega nomenclature of Rahm and Holman (1) which describes the position of the final double bond in the fatty acid molecule permits a simple description of the family relationships in the various fatty acid series, linoleate (ω6), linolenate (ω3), and oleate (ω9).

[2] See footnote 1; Witting, L. A., and M. K. Horwitt, Lipids *2*, 89 (1967).

[3] Activation Analysis Service, General Atomic Division of General Dynamics, San Diego, Calif.

selenite, sulfur amino acid, 0.4% *dl*-methionine, and tocopherol, 0.6 or 15.0 mg *d*-α-tocopheryl acetate/kg rat body weight/week were administered as indicated in the table headings in the next section. All groups were fed the appropriate rations containing the "dienoic" fat at the 1.2% level for two weeks and then transferred to the other fats described above.

Animals were sacrificed periodically by dislocation of the cervical vertebrae and the testes removed. Sections stained with hematoxylin and eosin were graded according to the system of Mason (15). Phospholipid phosphorus was determined by the procedure of Zilversmit and Davis (16). Phospholipids and neutral lipids were separated by thin-layer chromatography (17), and the fatty acid methyl esters were analyzed by gas-liquid chromatography (14). Sperm concentrations were roughly estimated during the last portion of the experiment by separating the tubules from the capsule and shaking half of a testis with a few glass beads for 30 min in 10 or 15 ml of bicarbonate-formalin solution and counting an aliquot of the resulting suspension (18). Using smaller or larger samples of testis gave comparable results when expressed in terms of sperm per total testes. Bieri and Andrews (19) found no alteration in the proportions of the various phospholipid classes in their study of testicular degeneration and reported that the changes in fatty acid composition were evident in all phospholipid classes. Analysis of individual phospholipids was therefore not considered necessary in the present investigation.

RESULTS

The preliminary experiments indicated that testicular degeneration was influenced by the nature of the dietary fat, groups I and III, Table I, and possibly, slightly by dietary selenium and methionine, groups I and II, but probably not by methionine alone, groups IV and V. A comparison of groups III, IV and V is of particular interest since the combinations of selenium, methionine and tocopherol fed were such that all three groups developed creatinuria as a sign of the onset of nutritional muscular dystrophy at approximately the same time (12, 12 and 13 weeks, respectively) (3). Testicular degeneration, however, proceeded much more rapidly in group III.

A group similar to group IV (Table I) was supplemented with 15 mg N,N'-diphenyl-paraphenylenediamine/kg rat body weight/week. Testicular degeneration was noted after approximately 25 weeks. The synthetic antioxidant, while not as biologically efficient as α-tocopherol, did delay testicular degeneration.

Testicular lipids from another group fed the "monoenoic" fat in the basal ration without added selenium and methionine or tocopherol supplementation were analyzed at regular intervals. Testicular degeneration occurred after 12-14 weeks, at which time there was a significant ($p < 0.001$), large (50%), net increase in the phospholipid arachidonate level. After 27-30 weeks the phospholipid content of the testes of the antioxidant deficient rats decreased to approximately one-half the control

TABLE I

Effect of Dietary Constituents on Testicular Degeneration in the Antioxidant-Deficient Rat

Group no.	Dietary fat	Supplementation: Selenium (ppm)	Supplementation: Methionine (%)	Supplementation: Tocopherol (mg/kg/wk)	Experimental period	Testicular degeneration (No. degenerating) / (No. examined)
I	"Monoenoic"[a] (7.5%)	0.13	0.4	0.4	38	0/2
					41	0/3
II		0	0	0.6	30-38	0/7
					41	2/3
III	"Trienoic" (7.5%)	0.13	0.4	0.4	18[b]	3/3
IV		0	0	1.2	30	0/3
					35	2/2
					38	1/2
					41	1/1
V		0	0.4	1.2	30	0/3
					35	1/1
					38	0/2
					41	2/2

[a] Monoenoic acids 89%, when fed at this level (7.5%), the diet contained 0.81% of calories as linoleate.

[b] In this group testes were not examined prior to 18 weeks. Since all samples were graded 5 on the 0–5 scale of testicular degeneration described by Mason (15), it was apparent that the onset of degeneration had occurred prior to this sacrifice period.

TABLE II

Testicular Weight and Phospholipid Content

Dietary fat		Dienoic (7.5%)				Dienoic (1.2%)		Trienoic (7.5%)		Polyenoic (7.5%)		Pooled control groups	
Supplementation													
Selenium (ppm)		0	0	0.13	0.13	0	0.13	0	0.13	0	0.13	[a]	[b]
Methionine (%)		0	0.4	0.4	0	0	0.4	0.4	0	0	0.4	[a]	[b]
Tocopherol (mg/kg/wk)		0	0	0	0	0	0	0	0	0	0	15	0.6
Weeks on experiment													
6	Testes (g)	3.0[c]	2.9	3.0	3.0	3.1	2.9	2.8	2.8	2.7	2.8	2.8±0.1 (19)[d]	2.8 (30)
	Phospholipid (mg/g)	14.2	14.0	14.0	13.8	11.9	13.1	14.4	13.7	15.1	14.8	13.9±0.2 (19)	13.9 (30)
8	Testes (g)	3.0	2.8	3.3	3.1	3.2	3.4	3.1	3.0	2.9	2.9	3.0±0.1 (16)	3.0 (30)
	Phospholipid (mg/g)	12.7	13.2	14.7	14.7	12.3	11.5	14.0	14.1	14.0	14.1	13.8±0.3 (16)	13.4 (30)
10	Testes (g)	3.4	2.8	3.4	2.9	3.4	3.0	3.1	3.0	3.0	3.1		
	Phospholipid (mg/g)	13.5	13.7	13.8	13.5	13.5	13.4	12.7	12.8	13.4	13.8		
11	Testes (g)											3.2±0.1 (17)	3.1 (30)
	Phospholipid (mg/g)											13.2±0.2 (17)	13.1 (30)
12	Testes (g)	2.3	2.2	2.2	2.9	1.6	2.4	2.6	2.7	3.2	2.7		
	Phospholipid (mg/g)	11.7	12.5	13.4	14.0	12.4	12.4	12.3	10.9	13.1	12.0		
13	Testes (g)											3.3±0.1 (16)	3.3 (30)
	Phospholipid (mg/g)											13.0±0.5 (14)	13.1 (18)
14	Testes (g)	2.3	2.0	1.2	1.3	1.4	1.6	2.0	2.0	3.0	3.2		
	Phospholipid (mg/g)	12.6	12.0	11.6	10.1	12.7	12.3	12.3	11.6	13.0	11.8		
16	Testes (g)											3.4±0.1 (15)	3.3 (30)
	Phospholipid (mg/g)											13.0±0.3 (15)	12.9 (30)

[a] Data from all animals supplemented with 15 mg *d*-α-tocopheryl acetate have been grouped together since no significant differences were noted attributable to dietary fat or selenium and methionine supplementation.

[b] Data from all animals supplemented with 0.6 mg *d*-α-tocopheryl acetate have been grouped together since no significant differences were noted attributable to dietary fat or selenium and methionine supplementation.

[c] Each value is the average of individual determinations on three animals.

[d] Average ± SEM, number of samples in parentheses.

level. At this time the arachidonate level of the testes expressed as micrograms of phospholipid arachidonate per gram wet weight of tissue was roughly comparable to that seen in the control animals, whereas there was an almost complete (90%) loss of docosapentaenoate (22:5ω6). These latter findings are in complete agreement with the data of Bieri and Andrews (19).

Testicular weight and phospholipid levels have been noted in Table II for animals fed a variety of experimental diets. Between the

TABLE III

Effect of Dietary Fat, Selenium, Methionine and Tocopherol on the Sperm Content of the Testes

Dietary fat	"Dienoic" (7.5%)				"Dienoic" (1.2%)		"Trienoic" (7.5%)		"Polyenoic" (7.5%)	
Selenium (ppm)	0	0	0.13	0.13	0	0.13	0	0.13	0	0.13
Methionine (%)	0	0.4	0.4	0	0	0.4	0.4	0	0	0.4
Tocopherol (mg/kg/wk)	0	0	0	0	0	0	0	0	0	0
Weeks on experiment	Total sperm, in millions, in testes of individual animals									
12					0	0	1	1	7	5
					0	4	7	3	10	7
					1			7	12	8
14	0	0	0	0	0	0	0	0	10	11
	1	1	0	0	0	0	1	0	20	12
	21	11	0		0	0	9	6	20	16

Data from all groups supplemented with 15 mg *d*-α-tocopheryl acetate/kg rat body weight/week were grouped together, since no significant differences were noted attributable to dietary fat or selenium and methionine supplementation. Sperm counts at 13 weeks 18 ± 1.0 (14)[a] and at 16 weeks 19 ± 0.5 (15).

Data from all groups supplemented with 0.6 mg *d*-α-tocopheryl acetate/kg rat body weight/week were grouped together, since no significant differences were noted attributable to dietary fat or selenium and methionine supplementation. Sperm counts at 13 weeks 20 ±2.0 (27)[a] and at 16 weeks 19 ± 1.1 (27).

[a] Average ± SEM, number of samples in parentheses.

TABLE IV

Histological Scoring[a] of Testicular Degeneration

Dietary fat (%)	"Dienoic" (7.5%)				"Dienoic" (1.2%)		"Trienoic" (7.5%)		"Polyenoic" (7.5%)	
Supplementation										
Selenium (ppm)	0	0	0.13	0.13	0	0.13	0	0.13	0	0.13
Methionine (%)	0	0.4	0.4	0	0	0.4	0.4	0	0	0.4
Tocopherol (mg/kg/wk)	0	0	0	0	0	0	0	0	0	0
Weeks on experiment										
8					0[b] (0,0,0)	0 (0,0,0)	0 (0,0,0)	0 (0,0,0)	0 (0,0,0)	0 (0,0,0)
10	0 (0,0,0)	0 (0,0,0)	0 (0,0,0)	0 (0,0,0)	1.0 (1,1,1)	2.0 (1,2,3)	1.0 (1,1,1)	1.0 (1,1,1)	1.0[c] (1,1,1)	1.0 (1,1 1)
12	3.0 (1,3,5)	2.3 (2,2,3)	2.3 (1,3,3)	2.5 (2,3)	4.3 (3,5,5)	3.0 (1,5)	3.5 (2,5)	1.7 (1,1,3)	0.7 (1,1,0)	1.0 (1,1,1)
14	3.7 (1,5,5)	3.3 (1,4,5)	4.0 (3,4,5)	5.0 (5,5)	4.7 (4,5,5)	3.7 (2,4,5)	2.3 (1,3,3)	2.0 (1,2,3)	1.0 (1,1,1)	1.0 (1,1,1)

[a] Graded on a 0-5 scale according to Mason (16).

[b] Values in parentheses are scores of individual animals, and the figures above these parentheses are the average scores.

[c] Testes from rats fed the polyenoic fat in the tocopherol-deficient diet were slightly "abnormal" in appearance but did not show rapid progressive degeneration, decrease in gross weight and sperm count, and alteration of the phospholipid fatty acid composition.

TABLE V

Fatty Acid Composition of Testicular Lipids as a Function of Dietary Fat[a]

Dietary fat	"Dienoic" (1.2%)	"Dienoic" (7.5%)	"Trienoic" (7.5%)	"Polyenoic" (7.5%)
	Phospholipid			
Fatty acid	(6)[b]	(49)	(6)	(10)
16:0	42.5	44.5	41.6	40.1
16:1	1.3	1.1	1.6	2.3
18:0	6.2	6.5	6.6	6.2
18:1	14.0	11.5	15.5	21.8
18:2	2.3	3.1	3.1	1.2
20:4	11.3	11.1	9.8	8.0
22:4	1.1	1.6	0.8	0.7
22:5[c]	13.0	10.4	8.6	5.7
22:5[d]	1.0	0.7	0.5	0.6
22:6	0.7	0.8	2.1	3.7
	Neutral Lipid			
Fatty acid	(4)	(39)	(14)[e]	(9)
16:0	32.8	29.6	31.7	35.0
16:1	13.2	9.7	13.5	11.7
18.0	2.2	2.7	3.4	2.8
18:1	42.7	35.3	39.6	37.2
18:2	2.4	10.7	2.8	0.9
18:3	—[f]	—	3.2	—
20:4	—	1.0	—	—
20:5[c]	1.0	2.0	1.4	0.8

[a] Analyses of testicular lipids are derived from adequately tocopherol supplemented (15 mg/kg rat/wk) control rats. Minor constituents, lauric and myristic acids and fatty aldehydes have been omitted.

[b] Number of animals in parentheses.

[c] 22:5ω 6 (Retention time 7.2 relative to stearate on ethylene glycol succinate polyester at 175° C).

[d] 22:5ω 3 (Retention time 8.0 relative to stearate on ethylene glycol succinate polyester at 175° C).

[e] Includes analyses from tissue of rats supplemented with 0.6 mg *d*-α-tocopheryl acetate/kg rat body weight/week, which also showed no evidence of testicular degeneration.

[f] Present, but comprising less than 0.8% of total fatty acids.

tenth and twelfth week of the experiment, a 12-52% decrease in gross testicular weight was noted in most of the groups not supplemented with α-tocopherol, the exception being those groups fed the lowest level of linoleate. The decrease in testicular weight was associated with a marked decrease in the estimated sperm concentration (Table III) and a slight decrease in phospholipid content expressed as mg phospholipid/g tissue (Table II). Upon excision, degenerative testes were characterized grossly by a purplish coloration in contrast to the normal pinkish-white tissue.

Histological evaluations of the extent of testicular degeneration on the 0-5 scale of Mason (15) are noted in Table IV. A grade of 2 in this system is indicative of complete sterility, and the higher grades represent various degrees of gross tissue degeneration. Frequently, two or three successive stages of degeneration were observed in different tubules of a single section and in a few extreme cases all stages of degeneration were apparent in the same histological section. The tabulated score represents the general impression of the grade typifying the entire slice and occasionally differs from the impression derived from fatty acid analyses, as will be noted later. During the stage of degeneration represented by the histological grade 1, there was a progressive decrease in spermatogenesis. A comparison of Tables III and IV reveals that testes receiving this score ranged from essentially normal to essentially zero in their sperm content. Relatively normal levels of sperm were present in all of the rats supplemented with 0.6 mg of

TABLE VI

Phospholipid Essential Fatty Acid Content of Testes as a Function of Diet and Stage of Degeneration[a]

Dietary fat	Tocopherol (mg/kg/wk)		Fatty acid (μg/g testes) 18:2	20:4	22:4	22:5	Total
Dienoic (1.2%)	15	(6)[b]	195 ± 8[c]	934 ± 51	93 ± 11	1041 ± 32	2263 ± 87
	0.6	(6)[d]	213 ± 4	1082 ± 119	111 ± 6	1188 ± 37	2594 ± 147
Stage 1[a]			150	1056	83	1014	2307
			208	1551	121	910	2790
Stage 2			185	1855	193	339	2572
Stage 4			283	1669	205	615	2772
Stage 5			183	1179	119	525	2006
			199	1624	157	415	2395
			161	1500	196	301	2158
Stage 1-5	0	(7)	207 ± 18	1451 ± 100[e]	152 ± 15[f]	—	—
Dienoic (7.5%)	15	(49)	275 ± 7	1051 ± 23	117 ± 6	968 ± 28	2415 ± 52
	0.6	(12)[d]	269 ± 10	1024 ± 67	101 ± 7	1045 ± 45	2442 ± 96
Stage 1			299	1382	111	1032	2844
			334	1453	117	1228	3132
Stage 2			241	1543	116	940	2840
Stage 3			148	1402	121	930	2641
Stage 4			260	1053	201	312	1826
Stage 5			307	1361	208	430	2306
			209	1417	166	282	2074
			280	1297	175	273	2025
			325	1070	201	238	1834
Stage 1-5	0	(9)	267 ± 20	1331 ± 55[g]	157 ± 14[f]	—	—
Trienoic (7.5%)	15	(6)	266 ± 14	929 ± 35	80 ± 7	813 ± 18	2018 ± 68
	0.6	(3)[d]	323 ± 54	982 ± 54	80 ± 17	1021 ± 135	2403 ± 91
Stage 3			283	1111	71	688	2153
			366	1735	164	463	2708
Polyenoic (7.5%)	15	(10)	103 ± 8	626 ± 81	53 ± 7	422 ± 26	1205 ± 29
	0	(5)[h]	63 ± 5	647 ± 37	20 ± 2	461 ± 32	1191 ± 63

[a] Graded on a 0–5 scale according to Mason (16). Values for fatty acids are for individual animals sacrificed at the designated stage. [b] Number of animals in parentheses. [c] Mean ± SEM. [d] Animals sacrificed at 16 weeks. [e] $p < 0.005$. [f] $p < 0.02$. [g] $p < 0.001$. [h] Animals sacrificed at 14 weeks.

d-α-tocopheryl acetate/kg rat body weight/week.

The effect of dietary lipid on the testicular phospholipid and neutral lipid fatty acid composition of the control (15 mg *d*-α-tocopheryl acetate/kg rat body weight/week) groups is shown in Table V. All comparisons have been related to the 1.2% "dienoic" fat-fed group. Raising the level of dietary linoleate from 1.10% to 6.3% of calories increased the linoleate content of the testicular neutral lipids markedly and of the phospholipids slightly, but did not otherwise alter the fatty acid composition. Feeding a diet supplying 1.18% of calories as linoleate and 3.75% of calories as linolenate, the 7.5% 'trienoic" fat-fed group, had little affect on testicular lipid fatty acid composition. A small amount of linolenate was noted in the neutral lipids and the docosahexaenoate (22:6ω3) content of the phospholipids increased by approximately 1.3%. In contrast to the observations in other tissues (14,20), the testicular levels of essential fatty acids were decreased only slightly (11%).

When the "polyenoic" fat was fed, 0.16% and 2.75% of calories were derived from linoleate and higher nonessential polyenoic acids (20:5, 22:5 and 22:6ω3), respectively. The essential fatty acid content of the testes was halved by this drastic limitation on linoleate intake, but the nonessential PUFA content of the testicular phospholipids rose only about 3%.

At the onset of testicular degeneration the net essential fatty acid content (18:2ω6 + 20:4ω6 + 22:4ω6 + 22:5ω6) of the testes, expressed as micrograms of phospholipid essential fatty acid per gram of tissue, increased, with the increase attributable to a large net increase in arachidonate (20:4ω6) (Table VI). Thereafter, a decrease in the total essential fatty acid level was observed as the docosapentaenoate (22:5ω6) level decreased to one-tenth or less of the normal level. Sterility was not associated with a simple decrease in 22:5ω6 per se since sperm counts were normal at lower levels of this fatty acid in the testes of the "polyenoic" fat fed group than the levels noted in the other groups during stages 1 and 2 of degeneration. Data obtained on the groups supplemented with 0.6 mg *d*-α-tocopheryl acetate/kg rat body weight/week for 16 weeks have been included in Table VI. There is some slight indication

that a rise in docosapentaenoate ($22:5_{\omega}6$) may precede other indications of testicular degeneration.

Addition of biologically available selenium and methionine to a diet known to be suboptimum in these nutrients by the criteria of growth apparently failed to influence greatly the course of testicular degeneration in any of the experiments reported herein.

DISCUSSION

The rate of testicular degeneration in the antioxidant-deficient rat is clearly related to the PUFA content of the tissue. A direct response to the level of dietary linoleate would seem to prevail, however, only in the limited region just short of testicular degeneration arising from essential fatty acid deficiency. This tissue appears to have not only a high priority upon the animal's supply of essential fatty acids, but also to be quite resistant both to the competitive inhibition of the synthesis of the higher essential PUFA noted in other tissues when nonessential polyunsaturated fatty acids are fed (14,20) and to the incorporation of the nonessential PUFA into its component lipids. Note in Table VI that when a fat was fed having a ratio of 17 parts nonessential PUFA to 1 part linoleate ("polyenoic" fat diet) the essential fatty acid content of the testes was limited by the low linoleate intake, but the higher essential PUFA made up about the same percentage of the total essential fatty acids as they did in the absence of the nonessential PUFA. This deficiency sign is thus distinct from nutritional muscular dystrophy in its response to only essential fatty acids and distinct from chick encephalomalacia in its failure to be "inhibited" by nonessential PUFA.

This deficiency sign is also different from all others that have been studied since significant modifications in response to alteration of dietary levels of selenium and sulfur amino acids were not noted. Considerable data have been included to document this lack of response.

Very little radioactivity, a few thousand dpm, was incorporated into the testicular PUFA 12 hr after the intraperitoneal injection of 0.2 μC of 1-C^{14}-acetate per gram rat. In a few experiments, approximately 7 times as much radioactive acetate was found to be incorporated into the testicular phospholipid pentaenoic acid fraction in the antioxidant-deficient rats as was incorporated into the corresponding fraction of tocopherol-supplemented rats. The data are inadequate, as proof, but are mentioned since they do resemble other data obtained in liver (4) and muscle (3). In both of these tissues an increased incorporation into the higher PUFA was noted despite the continued "disappearance" of the polyenoic acid from the tissue.

Testicular degeneration differs markedly, as noted above, from several other antioxidant-deficiency signs, but one specific phenomenon has been found to be common to muscle, liver, and testes of the antioxidant-deficient rat. In all three of these tissues adversely affected by antioxidant-deficiency there occurs a progressive net decrease in the most highly PUFA in the tissue and a net increase in its arachidonate content. One would be tempted to assume, therefore, that this phenomenon is related to the basic universal biochemical lesion in antioxidant-deficiency.

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The Effect of Antioxidant Deficiency on Tissue Lipid Composition in the Rat. IV. Peroxidation and Interconversion of Polyunsaturated Fatty Acids in Muscle Phospholipids

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ABSTRACT

It has been suggested that the net changes which take place in the composition of the muscle phospholipid fatty acids of the antioxidant-deficient rat represent the balance of two opposing processes. To compensate for (A) the preferential peroxidative destruction of the most highly polyunsaturated fatty acids in the tissue there occurs (B) an increase in the conversion of available precursors to the higher polyunsaturated fatty acids. Analysis of the data in terms of peroxidation kinetics indicated that the onset of creatinuria in one group after 3 weeks and in a second group after 7 weeks on an antioxidant-deficient diet occurred in both cases concomitant with the peroxidative "disappearance" of approximately 125 μg of phospholipid polyunsaturated fatty acid per gram wet weight of tissue or 2% of the total muscle phospholipid fatty acids.

INTRODUCTION

ONE COMPLEX PHENOMENON has been found to be common to three tissues, muscle (1), liver (2) and testes (3) in the antioxidant-deficient rat. A decrease in the most highly polyunsaturated fatty acid (PUFA) in the tissue lipids occurs with a concomitant net increase in arachidonate. Studies with C^{14}-acetate, however, indicate that an increased synthesis of not only arachidonate but also the higher PUFA occurs at the time their concentration is decreasing in the tissue. Bernhard et al. (4,5) have suggested that the increased net synthesis of arachidonate in the liver of the antioxidant-deficient rat results from a lack of tocopherol to regulate the oxidative reactions of fatty acid chain elongation and desaturation. The decrease in the essential docosapentaenoic acid (22:5ω6)[1] in the testes was thought by Bieri and Andrews (6) to result from the inability of the tocopherol-deficient rat to further metabolize arachidonate (20:4ω6). Witting and co-workers (1-3) have suggested that the compositional data are a composite reflecting the preferential peroxidative destruction of the most highly PUFA and accumulation of the product, arachidonate, closest to the available precursor, linoleate, formed in increased quantities in a homeostatic attempt to maintain normal membrane lipid fatty acid composition. This hypothesis based on a rather simple analysis of the rates of interconversion and "disappearance" of the PUFA in the muscle phospholipids is developed in the present paper.

EXPERIMENTAL

The experimental data used in the present analysis are derived from the first paper in this series (1).

RESULTS

Previous studies (7-9) of lipid peroxidation in vivo produced data in excellent accord with the kinetics of peroxidation of individual pure polyunsaturated fatty acids in vitro. It was found that as n in $CH_3(CH_2)_a(CH{=}CH\ CH_2)_n(CH_2)_b\ CO_2H$ increased from 2 to 6, the relative rates of fatty acid peroxidation increased according to the series 1:2:4:6:8 in vivo (7-9) and in vitro (10). These studies have suggested that the biological membrane, and oriented bimolecular lipid layer, may be treated as an isolated, hydrophobic region, containing the nonpolar portion of the long chain fatty acids, bounded by the polar, hydrophilic groups of the phospholipid molecules.

When the "disappearance" of PUFA from the tissue phospholipids of tocopherol-deficient animals was studied (1-3), it was anticipated that the rates of "disappearance" of the various individual PUFA should be determined by their relative susceptibility to peroxidation. This, however, was not found to be the case.

When the slopes of the curves showing the "disappearance" of the various muscle phospholipid fatty acids were determined for a group of rats fed on the "trienoic" fat in a diet without supplemental selenium, methionine or

[1] The omega nomenclature describes the position of the final double bond in the fatty acid molecule and thus permits a simple description of the family relationships in the various fatty acid series, linoleate (ω 6), linolenate (ω 3) and oleate (ω 9).

TABLE I

Changes in Fatty Acid Composition of Muscle Phospholipids in Tocopherol Deficient Rats

Fatty acid	Time required for PUFA levels to decrease to ½ of control level: Observed[a] (weeks)	Calculated[b] (weeks)	Corrected for interconversion[c] (weeks)
18:2	37	120	134
18:3	32	60	
20:4	– 30[d]	30	30
20:5	23	20	
22:5	60	20	
22:6	15	15	

[a] See Ref. 1 for details.

[b] Calculated from relative susceptibility to peroxidation *in vivo* (7) and based on the rate of disappearance of 22:6.

[c] See text.

[d] Increases to twice the control level in the time indicated.

tocopherol, the data in Table I were obtained. Docosahexaenoate decreased to one-half the level seen in control, tocopherol-supplemented, rats in 15 weeks, and arachidonate increased to twice the control level in 30 weeks. For a model system in which docosahexaenoate decreased to one-half of its initial level in 15 weeks, linoleate and arachidonate would be expected to decrease to one-half of their initial levels in 120 and 30 weeks, respectively. In the tissue, linoleate decreased much more rapidly and arachidonate was found to increase.

The observed data for arachidonate are shown in Fig. 1 A, solid line, and the expected peroxidative disappearance to one-half the control level in 30 weeks is indicated by the broken line. The algebraic difference between these lines corresponds to an increased net synthesis of arachidonate at the linear rate of 0.25% of the phospholipid fatty acids per week as indicated by the dotted line. In Fig. 1 B the observed data for linoleate are indicated by the solid line. When the observed level of the

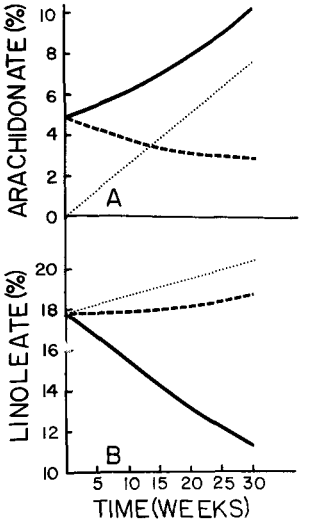

FIG. 1,A. Changes in arachidonate concentration in muscle phospholipid fatty acids of tocopherol-deficient rats. Observed data (solid line). Calculated data based on peroxidation kinetics (broken line). The differences between the observed and calculated data are indicated by the dotted line which is thought to describe increased synthesis of arachidonate.

1,B. Changes in linoleate concentration in muscle phospholipid fatty acids. Observed data on tocopherol-deficient rats (solid line) and on tocopherol-supplemented rats (dotted line). The broken line shows the observed data on tocopherol-deficient rats corrected for the arachidonate synthesis indicated in Fig. 1,A.

TABLE II

Peroxidative "Disappearance" and Interconversion of Linoleate and Arachidonate

Time (weeks)	Arachidonate observed[a] (%)	Arachidonate calculated[b] (%)	Arachidonate synthesis (%)	Linoleate observed tocopherol deficient[a] (%)	Linoleate corrected for synthesis of arachidonate[c] (%)	Linoleate observed tocopherol supplemented[d] (%)
0	5.1	5.1	0	17.8	17.8	17.8
5	5.7	4.4	1.3	16.6	17.9	18.2
10	6.4	3.9	2.5	15.4	17.9	18.6
15	7.2	3.4	3.8	14.3	17.9	19.0
20	8.1	3.1	5.0	13.3	18.1	19.4
25	9.1	2.8	6.3	12.4	18.4	19.8
30	10.3	2.8	7.5	11.5	18.7	20.3

[a] See Ref. 1 for details.

[b] Calculated on the basis of arachidonate decreasing to ½ the control level in 30 weeks.

[c] A small correction has also been included for the difference in molecular weight between linoleate and arachidonate.

[d] See Ref. 1 for details. The control arachidonate curve has a slope of zero whereas the control linoleate curve has a definite positive slope. The difference between columns 6 and 7 corresponds to a decrease of linoleate to ½ the control level in 134 weeks.

linoleate was corrected for arachidonate synthesis, the broken line, Fig 1 B, was obtained. The slope of this new curve corresponds to a decrease of linoleate to one-half the control level, dotted line, in 134 weeks. Note that in the control animals, linoleate (dotted line) progressively increased from approximately 18% to 20% of the phospholipid fatty acids over a period of 30 weeks. The experimental linoleate curve (broken line) corrected for arachidonate synthesis has a slight positive slope, increasing from approximately 18% to 19% in 30 weeks. This corrected experimental slope, however, is negative in comparison to the control slope. Actual numerical operations are noted in Table II.

No attempt was made to obtain an exact balance on the linolenate, eicosapentaenoate and docosapentaenoate series. The linolenate peak is poorly resolved in the gas chromatographic system used, ethyleneglycol succinate polyester, and the analytical error involved in determining components present at the 1-2% level is rather high. A comparison of the experimental and calculated curves for docosapentaenoate indicated an initial rate of increased synthesis comparable to the rate of increased arachidonate synthesis (0.12% per week). However, as linolenate levels fell to approximately 1%, the rate of extra docosapentaenoate synthesis progressively decreased.

It would appear, therefore, that observed changes in fatty acid composition may be resolved into two distinct competing processes. A balance of the type described above might, of course, be fortuitous. Comparable data have been obtained in three other experiments, however, and a second series of data is described below.

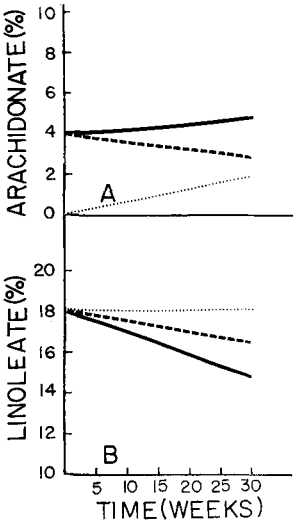

FIG. 2,A. Changes in arachidonate concentrations in muscle phospholipid fatty acids of tocopherol-deficient rats supplemented with selenium and methionine. Observed data (solid line). Calculated data based on peroxidation kinetics (broken line). The differences between the observed and calculated data are indicated by the dotted line, which is thought to describe increased synthesis of arachidonate.

2,B. Changes in linoleate concentration in the muscle phospholipid fatty acids. Observed data on tocopherol-deficient rats (solid line) and on tocopherol-supplemented rats (dotted line). The broken line shows the observed data on tocopherol-deficient rats corrected for the arachidonate synthesis indicated in Fig. 2,A.

TABLE III

Changes in Fatty Acid Composition of Muscle Phospholipids in Tocopherol-Deficient Rats Supplemented with Biologically Available Selenium and Methionine

Fatty acid	Time required for PUFA levels to decrease to ½ of control level		
	Observed[a] (weeks)	Calculated[b] (weeks)	Corrected for interconversion[c] (weeks)
18:2	103	256	225
18:3	—[d]	128	
20:4	−107[e]	64	64
20:5	—[d]	43	
22:5	56	43	
22:6	32	32	

[a] See Ref. 1 for details.

[b] Calculated from relative susceptibility to peroxidation *in vivo* (7) and based on the rate of disappearance of 22:6.

[c] See text.

[d] Does not differ significantly from control level and therefore cannot be said to be decreasing in concentration.

[e] Increases to twice the control level in the time indicated.

Data for rats fed the "trienoic" fat in a tocopherol-deficient diet supplemented with 0.13 ppm of selenium as sodium selenite and 0.4% sulfur amino acid, *dl*-methionine are shown in Table III. In this case docosahexaenoate decreased to one-half the control level in 32 weeks. Peroxidation kinetics suggest that linoleate and arachidonate should decrease to one-half the control levels in 256 and 64 weeks, respectively. Linoleate was observed to decrease more rapidly than expected and arachidonate was found to increase. As before, the experimental data, solid line, and the expected peroxidative "disappearance" for arachidonate, to one-half the control level in 64 weeks, broken line, have been plotted in Fig. 2 A. The algebraic difference between these lines corresponds to an increased net synthesis of arachidonate at the linear rate of 0.06% per week, as indicated by the dotted line. In Fig. 2 B the observed

TABLE IV

Correlation of Onset of Creatinuria and Fatty Acid Changes in the Muscle Phospholipids of the Tocopherol Deficient Rat

Fatty acid type	Level at start of experiment 1 (%)	Peroxidative decrease[a] at onset of creatinuria 3 weeks (%)	
Diene	17.8	0.3	
Triene	2.4	0.1	Total 2.0%
Tetraene	5.1	0.3	116 μg/g
Pentaene	5.8	0.4	
Hexaene	8.3	0.9	
Fatty acid type	**Level at start of experiment 2 (%)**	**Peroxidative decrease[b] at onset of creatinuria 7 weeks (%)**	
Diene	18.1	0.3	
Triene	1.6	0.1	Total 2.3%
Tetraene	5.1	0.4	133 μg/g
Pentaene	3.9	0.3	
Hexaene	8.7	1.2	

[a] Based on dienoate, trienoate, tetraenoate, pentaenoate and hexaenoate decreasing to ½ the control level in 120, 60, 30, 20 and 15 weeks, respectively. These rates of decrease are those which occur after the experimental data are corrected for interconversions of the polyunsaturated fatty acids. (See text)

[b] Based on dienoate, trienoate, tetraenoate, pentaenoate and hexaenoate decreasing to ½ the control level in 256, 128, 64, 43 and 32 weeks, respectively. These rates of decrease are those which occur after the experimental data are corrected for interconversions of the polyunsaturated fatty acids. (See text)

data for linoleate are indicated by the solid line. When the observed level of linoleate was corrected for arachidonate synthesis, the broken line (Fig. 2 B) was obtained. This new line, broken line, corresponds to peroxidative "disappearance" of linoleate reaching one-half the control level, dotted line, in 225 weeks.

When the peroxidative changes and the enhanced conversion of the lower PUFA to higher PUFA are resolved as described above a close correspondence to peroxidation kinetics is observed (columns 4, Tables I and III).

The gastrocnemius and quadriceps muscles of both tocopherol-deficient and tocopherol-supplemented rats were found to contain 5.8 mg of phospholipid fatty acids per gram wet weight of tissue (1). In the first group, Table I, creatinuria was noted to occur after 3 weeks as a sign of the onset of nutritional muscular dystrophy. It was previously shown that addition of only methionine to the diet did not alter the rate of production of creatinuria (1). In the second group supplemented with selenium and methionine, Table II, the production of creatinuria required 7 weeks. Onset of creatinuria in both cases occurred concomitant to a 2.0–2.3% or a 116–133 μg "peroxidative" decrease in phospholipid PUFA per g wet weight of muscle (Table IV). Addition of biologically available selenium to the diet of the antioxidant-deficient rat thus appears to decrease the rate of lipid peroxidation in vivo. It is not clear whether this action is attributable to selenium alone or if it depends on the combination of selenium and methionine. The possible mode of action of selenium and methionine has been discussed elsewhere (8).

DISCUSSION

The above treatment of the analytical data of muscle phospholipid fatty acid composition illustrates a possible explanation for the increased incorporation of C^{14}-labeled acetate into both arachidonic and pentaenoic acids at a time when the arachidonate level in the tissue shows a net increase and the pentaenoic acids show a net decrease. Since the higher polyunsaturated fatty acids appear necessary for normal cellular metabolism and membrane permeability, the increased conversion of the lower to the higher PUFA is viewed as a homeostatic mechanism. An effort is apparently made to maintain an at least partially acceptable membrane lipid composition. The resulting membrane may not be "normal" in terms of all biochemical processes but some degree of subcellular organization is maintained.

The enzymes for fatty acid chain elongation and desaturation are located in the mitochondria and endoplasmic reticulum (11) and presumably they utilize the fatty acids derived from the membrane lipids as their substrate in adjusting a purely local problem. Since the precursors available in the tissue phospholipids differ quantitatively from those available from the diet to the body as a whole, a definite bias is introduced favoring an accumulation of PUFA in the essential fatty acid series.

The analogy to the reactions noted in essential fatty acid deficiency are readily apparent. Low tissue phospholipid levels of the higher PUFA would be expected to result from the absence of dietary precursors. Instead, large quantities of oleate and palmitoleate are converted to eicosatrienoic acids (12), presumably in an effort to maintain a biologically acceptable membrane lipid composition. The resulting mitochondrial membranes are known, however, to be somewhat abnormal in their biochemical properties (13).

Subcellular architecture and organization is largely based on the separation of various regions or organelles from one another by lipid membranes. Normal biochemical function, of

course, requires a constant flux of materials across these fluid "barriers" with a concomitant expenditure of energy. Polar portions of the phospholipid molecules are compatible (hydrophilic) with the aqueous environment of the cytoplasm and it remains for the hydrophobic fatty acid chains to form the "barriers." The characteristics of these membranes are, in part, dependent on the nature of the PUFA incorporated into them. To the extent that this hydrophobic region constitutes a "barrier" to the constituents of the aqueous phase, the fatty acids must be considered an isolated lipid system (8,9). Prior to membrane disruption, the kinetics of lipid peroxidation within this isolated system appear to be comparable to the kinetics observed in model systems in vitro (8,9). It is perhaps for this reason that such a simple description of the changes in the fatty acid pattern of the muscle as that presented herein should be possible.

The above treatment is definitely not applicable to the experimental data from studies of hepatic or testicular lipids. Since all antioxidant-deficiency signs must be related initially to the occurrence of lipid peroxidation, we feel obligated to examine all data in terms of the kinetics of lipid peroxidation. By seeking to unify all the diverse data on the antioxidant-deficiency signs in terms of a common underlying phenomenon, a better understanding of the biological action of tocopherol may emerge.

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Relative Incorporation of Linoleic and Arachidonic Acid in Phospholipids and Triglycerides of Different Rat Tissues

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ABSTRACT

Fat-deficient rats were fed different amounts of methyl linoleate for increasing periods of time. The fatty acid composition of triglycerides and phospholipids of epididymal fat pad, epirenal fat depot, intestinal fat depot, liver, and the pool of heart, kidney, lungs and pancreas was determined. The distribution of the total amount of linoleic and arachidonic acid incorporated into phospholipids and triglycerides per rat was calculated. Phospholipids and triglycerides of depot tissues presented different fatty acid compositions.

Although the phospholipids of liver and the pool of heart, kidney, lung and pancreas specifically incorporated linoleic acid at the beginning they very rapidly attained a rather steady composition, whereas triglycerides went on incorporating the acid. The amount of linoleic acid incorporated into the phospholipids of depot tissues was rather small. The triglycerides undoubtedly contributed in the highest proportion to the total pool of linoleic acid. However, the highest proportion of arachidonic acid was found in the total pool of phospholipids.

The total amount of linoleic acid incorporated into the phospholipids was an approximately lineal function of the amount of phospholipids independent of period of administration and doses of methyl linoleate. Besides presenting two lineal functions of the amount of phospholipids, arachidonic acid showed a vertical increase coincident with a vertical decrease of the amount of eicosa-5,8,11-trienoic acid. At this period no change in the amount of the phospholipid was shown. This phenomenon is explained as a possible direct replacement of eicosatrienoic acid by arachidonic acid.

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INTRODUCTION

A GREAT NUMBER OF WORKS have been published concerning linoleic acid incorporation into different lipids and animal tissues. However, many of these studies involved feeding complex natural oils rather than pure esters. Thus, the results do not show the sole effect of linoleic acid but that of linoleic interrelated to the other acids of the diet. In many of these papers the composition of the fatty acids was studied in various tissues but no attention was paid to the distribution in the total mass of phospholipids and triglycerides. Further, many of the analyses of fatty acid composition of the depot fats included phospholipids. Therefore minor polyunsaturated constituents reported may belong to the phospholipids and not to the triglycerides.

In our previous works (1,2) a marked difference was observed in the incorporation of arachidonic acid into phospholipids and triglycerides when it was administered in the diet or synthesized by the animals from linoleic acid. The present work studies more precisely the distribution of linoleic and arachidonic acid between the phospholipids and triglycerides in relation to the time period of administration and amount of methyl linoleate administered to the rats. The variation of the composition of those phospholipids which accompany the depot fats is also studied.

EXPERIMENTAL

Methyl linoleate (90% pure) was obtained from sunflower seed oil by formation of urea adducts (3) and fractional distillation.

Administration of Methyl Linoleate and Separation of Lipids

Eighteen weanling male rats from the strain of this Institute were conditioned prior to administration of methyl linoleate by maintaining them for 70 days on a semisynthetic fat-free diet described elsewhere (4). These animals were divided into 6 groups of 3 animals each. One

TABLE I

Distribution of Phospholipids and Triglycerides in the Rat Tissues (mg per rat)

Animal groups	Fat depot						Liver		Heart + kidney + lung + pancreas	
	Epididymal		Epirenal		Intestinal					
	Ph[a]	Tg[b]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
1 d 0[c] t 0	28.7	924	14.6	523	32.0	472	194	72	100	76
2 d 100 t 1	28.5	650	15.0	155	26.7	206	225	38	125	84
3 d 100 t 2	28.0	965	14.1	300	19.5	385	177	77	263	146
4 d 100 t 15	45.1	1833	27.2	1557	23.6	514	197	147	202	223
5 d 400 t 15	31.9	2258	25.6	864	36.9	967	235	188	415	149
6 d 400 t 30	37.4	1455	20.3	1135	132.0	538	496	164	331	92

[a] Ph = Total phospholipids.

[b] Tg = Triglycerides.

[c] d = mg of methyl linoleate; t = period of administration in days.

group of animals (1 d0/t 0)[3] was then sacrificed. The remaining 5 groups were fed a fat-free diet supplemented with methyl linoleate prior to sacrifice. Groups 2 d 100/t 1, 3 d 100/t 2, and 4 d 100/t 15 were fed 100 mg of methyl linoleate daily for 1, 2 and 15 days, respectively, while groups 5 d 400/t 15 and 6 d 400/t 30 received 400 mg methyl linoleate daily for 15 and 30 days, respectively.

After the animals were killed, the livers, epididymal fat pads, intestinal fat depot, epirenal fat depot, heart, lungs, kidneys and pancreas were removed. Heart, lungs, kidneys and pancreas were put together in one pool for each group of animals. All the tissues were homogenized with chloroform-methanol (2:1 v/v) in a mechanical blendor and the lipids isolated by the procedure of Folch et al. (5). The lipid content was determined by vacuum evaporation of aliquots. Phosphorous was estimated by the procedure of Allen (6) and the phospholipid content calculated by multiplying the milligrams of phosphorous by the factor 25.

Separation of Phospholipids and Triglycerides

One gram of lipids of each group was dissolved in 30 ml of chloroform and the phospholipids were separated by adsorption on 10 g of activated silicic acid. Crude triglycerides were collected by washing the column with chloroform, and crude phospholipids were then eluted with methanol. The amount of triglycerides was determined by weighing an aliquot of the chloroform solution. In non-depot tissues these data included outstanding amounts of cholesterol and cholesterol esters. They were corrected by direct estimation of total cholesterol content. The values so calculated approximate the true triglyceride content and fulfill the requirements of the present work.

The triglycerides of the chloroform solution were purified by thin-layer chromatography (TLC) on Silica Gel G (Merck Darmstadt) in the dark. Chromatoplates 20 × 20 and 0.5 mm thickness were prepared according to Stahl. The solvent system used was petroleum ether (bp 60-80C)—ethyl ether-acetic acid 90:10:1 (v/v). Portions of the samples were run at the edges of the plate and triglycerides were located by iodine vapor. Corresponding areas not stained with iodine were scraped off and directly methanolyzed without elution using methanolic 3 *N* HCl for 3 hr at 60C.

Crude phospholipids were purified in a similar way. They were spotted and developed in the same type of chromatoplates and solvent. The phospholipids remained at the origin. They were scraped off and directly methanolyzed without elution.

[3] The number of the groups of rats is followed by *d* which denotes milligrams of methyl linoleate administered and *t* that denotes the days of administration. Ex. 2 $_{d}{}^{100}$ corresponds to group 2 that received 100 mg methyl linoleate for one day.

TABLE II

Fatty Acid Composition of Phospholipids and Triglycerides of Epididymal Fat Pad (per cent of total)

	Animal groups											
	d[b] 0		d 100		d 100		d 100		d 400		d 400	
Fatty[a] acids	1 t 0		2 t 1		3 t 2		4 t 15		5 t 15		6 t 81	
	Ph[c]	Tg[d]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
16:0[e]	23.4	20.8	35.0	23.1	20.1	27.1	23.0	28.5	31.6	26.0	32.8	28.7
16:1	15.5	14.5	17.2	14.0	11.1	13.9	11.4	14.6	11.8	14.7	6.8	10.0
18:0	3.3	2.5	2.4	2.4	4.3	2.2	6.4	2.2	6.5	1.8	9.0	3.1
18:1	52.5	55.3	40.8	50.3	46.4	48.8	44.2	47.1	35.3	46.2	33.4	36.4
18:2	0.7	1.2	0.8	1.8	1.2	2.7	4.4	1.9	7.7	7.8	14.0	14.6
20:3	0.6	——	0.3	——	0.7	——	0.5	——	0.3	——	0.3	——
20:4	0.3	0.5	0.2	0.5	0.6	0.3	0.5	0.7	0.8	0.5	3.9	3.3
22:5ω6	——	——	——	——	——	——	——	——	——	——	1.2	——

[a] Minor components not tabulated account for 100%.

[b] d = mg of methyl linoleate.
t = days of administration.

[c] Ph = Phospholipids.

[d] Tg = Triglycerides.

[e] The number before the colon denotes number of carbon atoms and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

Estimation of Fatty Acid Composition

Fatty acid composition was determined by gas-liquid chromatography in a Pye apparatus with ionization detector. The samples were run in 4 ft columns packed with 10% polyethylene glycol adipate on Chromosorb W (80-100 mesh) at 195C. Quantitation was carried out by triangulation and the results reported as area per cent. The quantitative results with Standard D of the NIH agreed with the stated composition data with a relative error of less than 10% for major components (> 10% of total mixture). The peaks of fatty acid methyl esters were identified by comparison with authentic standards. The check of the statistical error of the estimated fatty acid composition of the epididymal fat of group 6 (Table II) was not pooled but individually analyzed and standard deviations calculated. Standard deviations of 5.4, 2.4, 1.4, 4.5, 4.2 and 0.3 were found for palmitate, palmitoleate, stearate, oleate, linoleate and arachidonate, respectively.

RESULTS

The distribution of phospholipids and triglycerides by weight per rat in the tissues studied is shown in Table I. These data were necessary to calculate the total amount of linoleic and arachidonic acids bound to both lipids. They show, as it is well known, that phospholipids are found in depot tissues in very low proportion in relation to triglycerides. The highest increase in phospholipids was generally found in all the tissues when methyl linoleate was provided for long periods and in the highest amounts.

The fatty acid composition of the lipids of liver (Table V) and the pool of heart, kidney, lung and pancreas (Table VI) showed, as it is already known, that in phospholipids the unsaturation is higher than in triglycerides. Stearic, linoleic, eicosatrienoic and arachidonic acids were found principally in phospholipids, whereas palmitic, palmitoleic and oleic acid were in higher proportion in triglycerides. The changes found by linoleate feeding agree with our previous results (1). The typical essential fatty acid deficient composition was progressively modified by the linoleate. Linoleic acid was incorporated into both phospholipids and triglycerides whereas arachidonic acid increased pronouncedly only in phospholipids. The proportions of palmitoleic, oleic and eicosatrienoic acids were also altered.

The fatty acid composition patterns of phospholipids and triglycerides of the depot tissues, epididymal fat pad, epirenal fat depot and intestinal fat depot in relation to the amount of methyl linoleate in the diet and time of administration are shown in Tables II, III and IV. They indicate that the fatty acid composition is different for phospholipids and triglycerides. However, the difference is not as remarkable as the one shown for liver or the pool of heart, lung, kidney and pancreas. The difference is found mainly in the percentage of stearic acid which as usual is higher in phospholipids and the percentage of oleic and palmitoleic acids that is generally higher in triglycerides. The amount of the linoleic acid incorporated depended on the period of administration and amount of the methyl linoleate in the diet. It

TABLE III

Fatty Acid Composition of Phospholipids and Triglycerides of Intestinal Fat Depot (per cent of total)

	Animal groups											
	d[b] 0		d 100		d 100		d 100		d 400		d 400	
Fatty[a]	1		2		3		4		5		6	
acids	t 0		t 1		t 2		t 15		t 15		t 30	
	Ph[c]	Tg[d]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
16:0[e]	27.4	24.0	26.5	24.2	30.0	25.1	29.7	27.5	28.4	25.5	28.9	28.9
16:1	9.0	15.2	10.2	8.8	11.7	12.0	8.3	13.6	10.5	12.7	6.1	11.0
18:0	11.0	2.0	7.9	3.9	6.7	4.0	4.7	4.2	6.6	2.9	9.5	3.3
18:1	42.3	51.0	42.3	52.6	42.1	52.5	44.9	46.3	31.3	42.0	27.2	37.8
18:2	1.1	0.7	1.6	0.7	2.2	1.8	2.7	2.6	8.7	13.7	14.8	16.8
20:3	0.5	....	3.6	....	1.6	....	1.1	....	1.0	....	0.6	....
20:4	0.5	0.4	2.4	0.5	1.4	0.7	0.8	0.8	4.6	0.1	5.9	0.2
22:5ω6	....	....	....	....	....	....	....	....	....	....	0.9	....

[a] Minor components not tabulated account for 100%.

[b] d=mg of methyl linoleate.
t=days of administration.

[c] Ph=Phospholipids.

[d] Tg=Triglycerides.

[e] The number before the colon denotes number of carbon atoms and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

reached higher values in triglycerides than in phospholipids. The percentage of arachidonic acid was very low and the highest values were found with the longest periods and highest amounts of the administered methyl linoleate. The proportion was higher in phospholipids than in triglycerides but the differences were not very significant. The eicosatrienoic acid was a minor component in both classes of lipids, even in the fat-deficient rat. This is an important difference when compared to the phospholipids of liver or the pool of heart, kidney, lung and pancreas. In all these organs, as it is well known, the eicosa-5,8,11-trienoic acid was a major component in the fat-deficient rat and was rapidly decreased by the administration of methyl linoleate (Tables V and VI).

The relative percentage of linoleic acid in phospholipids and triglycerides in all the tissues, depended on the amount of methyl linoleate administered in the diet. It was generally higher in the phospholipids than in the triglycerides for any tissue for doses of 100 mg and short periods, but it was generally higher in triglycerides of all the tissues for doses of 400 mg (Table II to VI). However, when the amount incorporated was expressed by weight per rat it was shown (Table VII) that for depot tissues it was always significantly higher in triglycerides than in phospholipids. In the other tis-

TABLE IV

Fatty Acid Composition of Phospholipids and Triglycerides of Epirenal Fat Depot (per cent of total)

	Animal groups											
	d[b] 0		d 100		d 100		d 100		d 400		d 400	
Fatty[a]	1		2		3		4		5		6	
acids	t 0		t 1		t 2		t 15		t 15		t 30	
	Ph[c]	Tg[d]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
16:0[e]	27.2	28.7	32.8	29.7	33.7	30.1	33.1	28.4	32.0	29.4	31.6	28.0
16:1	17.5	18.4	10.3	17.2	13.5	16.3	7.5	9.5	9.5	11.1	8.5	7.6
18:0	2.5	3.0	6.9	3.2	3.9	3.8	5.4	3.3	4.9	4.0	5.8	3.4
18:1	47.0	58.9	40.5	54.2	43.8	52.0	45.2	53.4	34.8	37.6	37.5	42.5
18:2	1.0	0.7	2.1	0.8	1.1	1.7	4.6	2.4	8.4	11.5	11.6	16.1
20:3	0.2	....	0.4	....	0.4	....	0.7	....	0.6	....	....	....
20:4	0.3	0.3	1.3	0.4	0.2	0.3	2.9	0.1	1.1	0.5	0.4	0.5
22:5ω6	....	....	....	....	....	....	....	....	....	....	....	....

[a] Minor components not tabulated account for 100%.

[b] d=mg of methyl linoleate.
t=days of administration.

[c] Ph=Phospholipids.

[d] Tg=Triglycerides.

[e] The number before the colon denotes number of carbon atoms and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

TABLE V

Fatty Acid Composition of Phospholipids and Triglycerides of Liver Lipids (per cent of total)

	Animal groups											
	d[b] 0		d 100		d 100		d 100		d 400		d 400	
Fatty[a]	1		2		3		4		5		6	
acids	t 0		t 1		t 2		t 15		t 15		t 30	
	Ph[c]	Tg[d]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
16:0[e]	15.2	24.9	19.6	26.8	21.1	25.4	17.4	31.1	17.9	26.7	16.9	27.3
16:1	6.0	14.6	3.7	13.7	4.2	9.5	2.9	8.1	2.5	10.2	1.6	5.1
18:0	24.8	3.4	26.7	3.7	24.6	2.5	22.3	2.4	25.4	2.4	26.3	3.1
18:1	25.0	49.2	24.2	47.7	19.6	50.1	19.0	42.5	8.9	34.8	8.2	30.6
18:2	2.1	1.3	6.7	2.5	5.5	2.1	4.6	4.6	9.0	15.9	11.5	21.2
20:3	11.3	0.6	8.6	0.5	7.9	0.3	1.8	....	0.9	....	0.3	....
20:4	7.4	....	7.7	0.6	11.7	0.6	22.0	3.9	26.3	3.1	26.5	4.1
22:5ω6	0.2	....	0.3	....	0.7	....	2.5	....	2.9	....	3.5	....

[a] Minor components not tabulated account for 100%.

[b] d=mg of methyl linoleate.
t=days of administration.

[c] Ph=phospholipids.

[d] Tg=Triglycerides.

[e] The number before the colon denotes number of carbon atoms and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

sues it was generally slightly higher in phospholipids. In most cases this distribution might be partially considered as the result of the relative amounts of triglycerides and phospholipids in both kinds of tissues.

The percentage of arachidonic acid was generally higher in phospholipids than in triglycerides of all the tissues and it increased with the period of time and amount of methyl linoleate administered. However, in the depot tissues the amount of arachidonic acid expressed per rat (Table VIII) was generally higher in the triglycerides than in the phospholipids. Although this distribution is undoubtedly again the result of the preponderant amount of triglycerides over phospholipids in the depot tissues, it can not be explained as a simple partition between both lipids. The relationships between the ratio $\frac{\text{Tg}}{\text{Ph}}$ in Table I and the ratio $\frac{\text{20:4 Tg}}{\text{20:4 Ph}}$ in Table VIII show a significant selectivity of arachidonic for the phospholipids.

When the total amount of linoleic, arachidonic and eicosatrienoic acid incorporated into the phospholipids of all the tissues analyzed

TABLE VI

Fatty Acid Composition of Phospholipids and Triglycerides of the Pool of Heart, Kidney, Lung and Pancreas (per cent of total)

	Animal groups											
	d[b] 0		d 100		d 100		d 100		d 400		d 400	
Fatty[a]	1		2		3		4		5		6	
acids	t 0		t 1		t 2		t 15		t 15		t 30	
	Ph[c]	Tg[d]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
16:0[e]	17.7	21.6	19.6	27.4	29.4	23.7	26.1	25.2	23.5	25.1	20.8	19.4
16:1	3.7	20.2	4.0	6.3	3.9	8.5	3.7	9.1	2.9	9.2	3.0	9.2
18:0	18.3	2.6	19.7	4.9	17.1	7.5	19.2	5.1	19.4	5.3	19.0	5.5
18:1	16.3	46.1	21.2	51.0	19.3	44.1	14.1	48.5	12.3	48.0	12.0	48.9
18:2	2.0	3.4	4.4	3.7	6.0	4.1	4.8	4.5	8.8	7.6	9.4	11.3
20:3	12.7	0.6	11.0	....	7.2	....	2.2	....	1.1	....	0.4	....
20:4	10.9	0.3	12.4	0.5	9.5	2.7	19.7	1.0	21.0	1.0	23.6	0.8
22:5ω6	0.4	....	0.5	....	0.8	....	1.1	....	1.0	....	1.4	....

[a] Minor components not tabulated account for 100%.

[b] d=mg of methyl linoleate
t=days of administration.

[c] Ph=phospholipids.

[d] Tg=Triglycerides.

[e] The number before the colon denotes number of carbon atoms and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

TABLE VII

Weight of Linoleic Acid Incorporated into the Phospholipids and Triglycerides of the Rat Tissue (mg per rat)

Animal groups	Fat depot						Liver		Heart+kidney+lung +pancreas	
	Epididymal		Epirenal		Intestinal					
	ph[a]	Tg[b]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
1 d 0[c] t 0	0.1	11.6	0.1	3.6	0.2	3.3	2.8	0.9	1.4	2.7
2 d 100 t 1	0.2	11.1	0.1	1.2	0.7	1.4	10.5	0.9	3.8	2.7
3 d 100 t 2	0.2	25.2	0.2	4.8	0.2	6.6	6.8	1.5	11.1	5.6
4 d 100 t 15	1.4	32.7	0.9	35.1	0.4	12.5	6.3	6.4	6.7	9.5
5 d 400 t 15	1.7	166.7	1.5	94.9	2.2	125.7	14.6	28.4	25.5	10.8
6 d 400 t 30	3.6	202.6	1.6	174.2	13.6	86.2	39.5	33.1	21.5	9.9

[a] Ph=Phospholipids.

[b] Tg=Triglycerides.

[c] d=mg of methyl linoleate; t=period of administration in days.

was plotted versus the amount of phospholipids, typical curves were found (Fig. 1). The amount of linoleic acid was a lineal function of the amount of phospholipids independent from time and doses. The equation was $y = 0.12x-42.5$ in which y stands for milligrams of linoleic acid and x the milligram of phospholipids. On the contrary the amount of arachidonic acid incorporated showed two curves with different slopes. The first one showed a smoother slope and a similar angular coefficient than the linoleic curve. The equation found was $y' = 0.12x-38.9$ where y' stands for the milligrams of arachidonic acid found in phospholipids. The

TABLE VIII

Weight of Arachidonic Acid Incorporated into the Phospholipids and Triglycerides of the Rat Tissues (mg per rat)

Animal groups	Fat depot						Liver		Heart+kidney+lung +pancreas	
	Epididymal		Epirenal		Intestinal					
	Ph[a]	Tg[b]	Ph	Tg	Ph	Tg	Ph	Tg	Ph	Tg
1 d 0[c] t 0	0.05	4.1	0.03	1.7	0.11	1.9	9.9	0.4	7.6	0.3
2 d 100 t 1	0.03	3.1	0.02	0.6	0.44	1.0	12.0	0.2	10.8	0.4
3 d 100 t 2	0.10	3.1	0.14	1.0	0.19	2.5	14.4	0.2	17.4	3.7
4 d 100 t 15	0.16	12.7	0.55	2.1	0.14	1.3	30.2	5.5	27.6	2.2
5 d 400 t 15	0.19	11.0	0.19	4.0	1.16	0.7	43.0	5.5	60.6	1.5
6 d 400 t 30	1.01	14.4	0.06	5.1	5.42	1.1	90.7	6.5	54.3	0.7

[a] Ph=Phospholipids.

[b] Tg=Triglycerides.

[c] d=mg of methyl linoleate; t=period of administration in days.

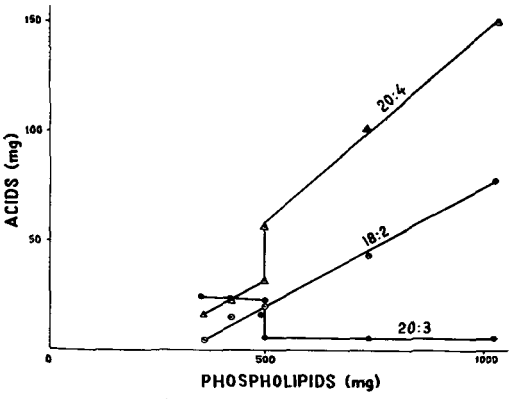

FIG. 1. Relationship between the amount of linoleic, arachidonic and eicosatrienoic acids incorporated in the phospholipids and the amount of phospholipids in the pool of tissues.

second slope was steeper and showed a lineal equation of the form $y' = 0.18 \times -30.4$. A vertical jump was shown when the amount of phospholipids was of the order of 500 mg and corresponded to the 2- to 15-day period of linoleate administration. The vertical increase was coincident with a vertical decrease of eicosatrienoic acid.

When the equations were calculated in a molar basis it was possible to show that a steady molar relation of 0.45 moles of arachidonic acid per mole of phospholipid was attained as compared to 0.34 for linoleic acid. Both acids are mainly bound to the β position. Therefore both acids together esterify 80% of the β hydroxy group of phospholipids. These results corroborate our previous work (2).

DISCUSSION

The administration of methyl linoleate to fat-deficient rats evokes a series of changes in the fatty acid composition of the tissues. These changes are undoubtedly related to the nature of the carrier that transports the fatty acid, to the subsequent transformation that fatty acids may suffer in the different tissues and to the kind of lipids that constitute these tissues. The linoleate is transported by chylomicrons from the intestine to the different tissues. In the liver it is converted into arachidonic acid and other unsaturated acids following a series of oxidative desaturations and elongations. In Tables V and VII it is shown that dietary linoleic acid is immediately incorporated into the phospholipids of liver and it increases before any change in the triglycerides is found. This effect may be attributed to a selective incorporation of linoleic acid in the phospholipids and not to a diluting effect. After this short period of approximately 48 hr for doses of 100 mg per rat, the linoleic acid incorporated by the phospholipids seems to reach an equilibrium, since the percentage composition attained a relatively constant level. This is shown in Table V and in our previous works (1,2). Then, the incorporation of linoleic acid into the triglycerides of these tissues starts increasing and accumulates markedly in triglycerides (Tables V and VII). The amount of linoleic acid in the diet may, however, modify the conditions of equilibrium, and higher percentages were found either in phospholipids or in triglycerides when higher doses were provided.

The incorporation of linoleic acid into the depot tissues followed another pattern and from the beginning the percentage in phospholipids did not differ very much from the percentage in triglycerides (Tables II to IV). However, the incorporation was quantitatively higher in triglycerides than in phospholipids (Table VII).

Generally, the total synthesis of phospholipids would be the preferable pathway for linoleic acid incorporation in comparison with direct transacylation (7). This hypothesis would be stressed by the practically lineal relationship found between the amount of phospholipid and the amount of linoleic acid incorporated into these lipids (Fig. 1). The step where linoleic acid is preferentially diverted to phospholipid in certain tissues can not be precisely identified for the moment. However, considering Kennedy's pathway of triglycerides and phospholipid synthesis, it may be that diglycerides containing linoleic acid are more selectively converted into phospholipids than into triglycerides. It is also possible that a superimposed transacylation reaction[7] would be responsible for such effect.

Arachidonic acid synthesized from linoleic acid in the liver is principally incorporated into the β position of phospholipids as shown in our previous work (2). It was found especially in liver and in the pool of heart, kidney, lung and pancreas; but very little was found in phospholipids of adipose tissues. It was also very slightly incorporated into the triglycerides of all the tissues (Table VIII).

For the moment we have been unable to desaturate linoleate into γ-linolenate using adipose tissue microsomes in vitro.[2] Therefore the measurable amount of arachidonic acid found in the triglycerides of the depot tissues, in spite

[2]Peluffo, R. O. and R. R. Brenner, to be published.

of the low percentage found in these lipids, probably comes mainly from the liver. The high amount of triglycerides in adipose tissues undoubtedly decreases the percentage of the acid by dilution. However, the low percentage of arachidonic acid also found in the phospholipids of the same tissues requires an additional explanation. Whereas linoleic acid was mainly transported in the chylomicrons, arachidonic acid synthesized in the liver would be transported in the phospholipids. Therefore the arachidonic acid available for adipose tissue may be quite limited in relation to the linoleic and other acids.

In 1963 (4) we postulated that the arachidonic acid might displace eicosa-5,8,11-trienoic acid from the β position of ethanolamine and choline phospholipids of fat-deficient rats through a direct transcylase reaction of the kind described by Lands and Merkle (7,8). The importance of this kind of reaction in phospholipid synthesis was stressed by the discovery of Van Deenen (9) that liver possesses a phospholipase able to deacylate either α or β carbon of phospholipids. This hypothesis easily explained the existence of the correlative curves found in our previous work (2) beween the incorporation of arachidonic acid and the depletion of eicosa-5,8,11-trienoic from the β position of the phospholipids of liver versus time when arachidonic acid was supplied to the diet of fat-deficient rats. It may also explain the similar curves found in heart (1) when either linoleic or arachidonic acid were administered to the diet. However, when methyl linoleate was fed (2) no correlative curves were found in the liver for the arachidonic increase and eicosatrienoic acid decrease. At that time this effect was explained by the existence of competitive reactions in the synthesis of arachidonic and eicosatrienoic acid. Nowadays this type of competition is confirmed by the discovery that linoleic and oleic acid compete in vitro for the same oxidative dehydrogenase which produces γ linolenic and octadeca-6,9,-dienoic acid from the corresponding substrates (10). However, when in the present experiment the amount of arachidonic and eicosatrienoic acid incorporated into the phospholipids was plotted versus the amount of phospholipids two typical curves were found (Fig. 1). Although part of the curves showed lineal functions between the arachidonic acid content and the amount of phospholipid, favoring the hypothesis of a total direct synthesis of phospholipids containing arachidonic acid molecules, the existence of a vertical increase in the arachidonic acid content correlative to a vertical decrease in eicosatrienoic acid without any change in the amount of phospholipid would corroborate the other hypothesis according to which arachidonic acid could directly displace eicosatrienoic acid from phospholipids. It is interesting to remark in these curves that once the eicosatrienoic acid was eliminated, the arachidonic acid increased again following a lineal function of the phospholipid content.

In consequence, gathering all these pieces of information, including either in vivo or in vitro experiments, we may postulate that the administration of linoleic acid to fat-deficient rats immediately interrupts the synthesis of eicosa-5,8,11-trienoic acid from oleic acid by competitive inhibition at the level of the oxidative desaturation of oleic into octadeca-6,9-dienoic acid and very probably at other steps including elongation.

Linoleic acid seems to be incorporated into the phospholipids mainly through the total synthesis of the lipids, but it does not displace eicosatrienoic acid. The direct displacement of eicosa-5,8,11-trienoic acid from the phospholipids would be evoked by the arachidonic acid either synthesized from linoleic acid or provided in the diet. This effect would be independent from the interruption of eicosatrienoic acid synthesis. A direct β transacylase reaction of the type described by Lands and Merkle (7) may very probably be responsible for such a displacement. However, arachidonic acid incorporation into phospholipids would also follow Kennedy's pathway of total phospholipid synthesis. This would arise from the type of the curve of arachidonic acid (Fig. 1) once eicosatrienoic acid was eliminated from the lipids.

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Metabolic Conversions and the Positional Distributions in Liver Lecithin of Some Unnatural Dienoic Acids in the Rat

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ABSTRACT

The metabolism of 9,15-octadecadienoate, 12,15-octadecadienoate and 7,13-eicosadienoate was investigated in the fat-deficient rat. The liver lecithins of those animals receiving 9,15- and 12,15-octadecadienoate, as well as the animal remaining on the fat-deficient control, were isolated. The fatty acid distributional pattern was determined by the use of lecithinase A. All three of the experimental dienoic acids were incorporated into tissue lipids. None of the acids, however, was converted to longer chain polyunsaturated fatty acids. The 9,15-octadecadienoate was esterified almost exclusively to the β-position of lecithin, whereas 12,15-octadecadienoate was about equally distributed between the α- and β-positions.

INTRODUCTION

THE BIOSYNTHESIS of polyunsaturated fatty acids (PUFA) may be visualized as a sequence of two carbon elongation and desaturation reactions. In pioneering work Mead and Howton (1) showed that linoleate (18:2ω6) is converted to arachidonate (20:4ω6) in the rat via the following pathway: 18:2ω6$\rightarrow$18:3ω6$\rightarrow$20:3ω6$\rightarrow$20:4ω6. Subsequent studies showing that the levels of 22:4ω6 and 22:5ω6 in tissue lipids are elevated when either 18:2ω6 or 20:4ω6 are fed to rats suggest that linoleate is likewise the initial precursor of these two acids (2). In a similar manner, oleate, palmitoleate and linolenate each serve as the initial unsaturated precursor for an independent family of PUFA (3–6). Although the quantity of each family of PUFA found in tissue lipids reflects the composition of the dietary lipid (7), there is no direct crossover in metabolism between the four families of fatty acids (8). For example, members of the ω6 family are not converted to ω3 acids in animals.

Although the pathways of PUFA biosynthesis are quite firmly established, little is known about either the enzymes involved in these interconversions or the structural features in the fatty acid molecule that are essential for chain elongation and desaturation. In an attempt to gain further insight into the type of structure which can be converted to longer chain PUFA, we wish to report our metabolic studies with rats on the following three unnatural dienoic acids: 9,15-octadecadienoate, 12,15-octadecadienoate and 7,13-eicosadienoate.

EXPERIMENTAL PROCEDURE

Fatty Acid Esters

The total synthesis of 7,13-eicosadiynoic acid used in this study was carried out independently by Gunstone and Sykes (9). The diynoic acid was converted to the methyl ester with diazomethane (10). The methyl 7,13-eicosadienoate so obtained was reduced to methyl 7,13-eicosadienoate with Lindlar's catalyst (11). The resulting compound was pure as judged by gas-liquid chromatography (GLC) and contained 6.0% isolated *trans* double bonds as determined by the infrared spectrum.

The selective reduction of methyl linolenate, followed by countercurrent fractionation of the resulting octadecadienoate isomers, has been described previously (12). The 9,15-octadecadienoate was pure as judged by GLC and contained 5.5% isolated *trans* double bond. It was not possible to separate the 9,12-octadecadienoate from the 12,15-isomer. The mixture contained 65.5% of the 9,12-isomer and 34.5% of 12,15-octadecadienoate as determined by GLC. The mixture contained 8.7% isolated *trans* double bond.

Nutritional Study

Twenty male weanling rats of the Sprague-Dawley strain were kept on a fat-deficient diet (13) for about 3 months. The animals were then subdivided into five groups of four animals each. Each animal in groups 1–3 was fed by syringe 200 mg of one of the experimental esters each day for a period of 5 days. The fourth group was given an equal amount of methyl linoleate, and the fifth group was maintained on the fat-deficient control diet.

The amount required for feeding the 9,12–12,15 mixture was 530 mg in order that each animal receive 200 mg of the 12,15-octadecadienoate each day. The mixture was fed in three equal aliquots each day, whereas those animals receiving 200 mg per day of other esters were given 100 mg twice daily.

Lipid Extraction and Fatty Acid Analysis

The animals were sacrificed by ether anesthesia. The livers were quickly removed and immediately frozen in isotonic saline until they could be analyzed. The lipids from each liver were extracted with chloroform:methanol, 2:1, as described by Folch et al. (14). An aliquot of each extract was taken to dryness under nitrogen and interesterified with methanolic HCl as described by Stoffel et al. (15). The methyl esters were analyzed on a Barber-Colman model 10 GLC apparatus, which was equipped with an argon ionization detector using a 210 cm glass column packed with 20% ethylene glycol succinate on Gas-Chrom P, 80–100 mesh, and a flow rate of 60 ml of argon per minute at 185C. The individual methyl esters were identified by equivalent chain length (16) and with internal standards. Quantification was done by triangulation and data are reported as area percent.

Isolation and Analysis of Lecithin

Lecithin was isolated from the rat livers of only those animals fed methyl-9,15-octadecadienoate, the 9,12–12,15-octadecadienoate mixture and the fat-deficient control group. The chloroform:methanol extract of the livers of the animals within each group was pooled and taken to dryness under reduced pressure. The lipids were dissolved in small volumes of chloroform:methanol, 2:1, and streaked on preparative thin-layer plates. The plates were developed with chloroform:methanol:water, 65:25:4 (v/v/v), after which the lipids were detected by spraying with 0.2% 2′, 7′-dichlorofluorescein in 95% ethanol. The fraction corresponding to lecithin was scraped from the plate and extracted. A portion of the lecithin was interesterified, and another aliquot was treated with snake venom *(Crotalus adamanteus)* as described by Long and Penny (17). The resulting lysolecithins and free fatty acids were separated by preparative thin-layer chromatography (TLC), scraped from the plate and immediately interesterified. The purity of lecithin isolated from the preparative TLC plates, as well as the completeness of the snake venom reaction, were monitored by TLC.

RESULTS AND DISCUSSION

Metabolism of Dienoic Acids

It has generally been assumed that feeding studies must be carried out for 50 to 100 days in order to observe meaningful changes in the fatty acid composition of tissue lipids. Recently studies by Schlenk et al. (18) and also by Rahm and Holman (2,19) have shown that the tissue lipid fatty acid pattern of essential fatty acid (EFA) deficient rats responds to dietary fats within 5 days.

The three experimental dienoic acids might be metabolized in one or more of the following three ways: 1) The three dienoic acids might be incorporated directly into tissue lipids without being chain elongated or desaturated. 2) If the rat is able to insert a double bond into position 9 of 12,15-octadecadienoate or into position 12 of 9,15-octadecadienoate, the resulting acid would be linolenate. If either of these desaturations occurred, the linolenate would enter the $\omega 3$ metabolic pathway (4) and an increase in the levels of the $\omega 3$ fatty acids in the tissue lipids should be observed. In a similar manner, desaturation at position 10 of 7,13-eicosadienoate would yield $20{:}3\omega 7$. If this interconversion proceeded, an increase in the levels of the $\omega 7$ fatty acids should result. 3) Elongation of the fatty acid might be possible without introduction of a double bond. The elongated acids could then be incorporated into tissue lipids or desaturated to give trienoic acids not containing the normal skipped unsaturation pattern. For example, if this pathway were operative, 9,15-octadecadienoate would be first converted to 11,17-eicosadienoate and subsequently either incorporated directly into tissue lipids or desaturated to 8,11,17-eicosatrienoate and then incorporated.

The averaged fatty acid compositions of the liver fatty acids and standard deviations are given in Table I. As is evident from the data, all three dienoic acids are incorporated into tissue lipids. However, their level of incorporation is considerably lower than observed for linoleate when this acid was the dietary variable. In those animals receiving linoleate, the level of this acid in liver lipids was 7.6% of the total liver fatty acids. In contrast, when 7,13-eicosadienoate and 9,15-octadecadienoate were the dietary variables, the respective percentage in liver lipids was 2.3% and 2.7%. An even more striking difference was observed with the 9,12–12,15-octadecadienoate mixture. Although the ratio of the 9,12–isomer to the 12,15–isomer in the diet was 65.5 to 35.5, or about 2 to 1, the ratio of the 9,12–isomer to

TABLE I

Fatty Acid Composition of Liver Lipids
(Average area percentage by GLC, and standard deviation)

	Dietary component				
Fatty acid	*Fat-free Control diet*	*9,12–18:2*	*9,12 and 12,15–18:2*	*9,15–18:2*	*7,13–20:2*
14:0	1.2 ± 0.3	0.7 ± 0.4	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
16:0	20.9 ± 2.0	21.0 ± 1.1	16.9 ± 1.7	19.9 ± 1.7	21.2 ± 1.4
16:1	13.3 ± 1.1	7.1 ± 1.0	6.1 ± 3.0	10.1 ± 0.6	10.2 ± 0.5
18:0	11.0 ± 2.3	16.4 ± 0.5	16.1 ± 3.4	11.5 ± 0.3	13.5 ± 2.2
18:1	36.4 ± 3.4	22.9 ± 3.2	21.2 ± 6.2	34.4 ± 1.7	32.8 ± 2.7
9,12–18:2	*1.5 ± 0.3*	*7.6 ± 0.8*	*10.7 ± 0.8*	1.5 ± 0.5	1.5 ± 0.3
9,15–18:2	— —	— —	— —	*2.7 ± 0.1*	— —
12,15–18:2	— —	— —	*2.2 ± 0.4*	— —	— —
18:3ω3	*0.4 ± 0.3*	0.4 ± 0.1	*0.4 ± 0.1*	*0.5 ± 0.2*	0.4 ± 0.1
7,13–20:2	— —	— —	— —	— —	*2.3 ± 0.4*
20:3ω9	*9.3 ± 1.8*	*3.7 ± 1.0*	2.9 ± 0.3	*10.3 ± 0.3*	10.6 ± 1.5
20:3ω6	1.0 ± 0.5	1.7 ± 0.1	1.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.1
20:4	*2.7 ± 0.9*	14.8 ± 0.9	16.3 ± 4.4	2.7 ± 0.3	*3.3 ± 0.7*
20:5ω3	*0.3 ± 0.2*	0.3 ± 0.2	*0.2 ± 0.1*	*0.4 ± 0.1*	0.3 ± 0.2
22:4ω6	0.4 ± 0.3	0.2 ± 0.1	0.5 ± 0.3	0.6 ± 0.2	0.3 ± 0.2
22:5ω6	0.4 ± 0.2	2.2 ± 0.3	2.2 ± 1.6	0.4 ± 0.3	0.5 ± 0.3
22:5ω3	Trace	Trace	Trace	Trace	Trace
22:6ω3	*1.1 ± 0.3*	1.1 ± 0.5	*2.0 ± 0.3*	*3.6 ± 1.9*	1.5 ± 0.3

the 12,15–isomer in liver lipids was about 5 to 1.

As discussed in the second hypothesis, conversion of 9,15– and 12,15–octadecadienoate to linolenate should result in an increase in the levels of 18:3ω3, 20:5ω3, 22:5ω3 and 22:6ω3 compared with the fat-deficient control animals. The experimental results indicate that the level of 22:6ω3 is slightly elevated when the animals received either 9,15– or 12,15–octadecadienoate. However, a corresponding elevation of 18:3ω3, 20:5ω3 and 22:5ω3 was not observed. If appreciable conversion to linolenate had occurred, the levels of these ω3 acids should have increased in a more dramatic fashion. For example, when fat-deficient rats received 100 mg of methyl linolenate per day for a period of 5 days, the respective levels of 22:5ω3 and 22:6ω3 rose about 20– and 6–fold over deficient controls (2). When an animal is maintained on a fat-deficient diet, the principal PUFA which increases is 20:3ω9 (20). The level of this acid is repressed when the animal is fed acids of either the ω3 or ω6 family. As shown in Table I, the content of 20:3ω9 remains at the same level as for the fat-deficient controls when the dietary acid was 9,15–octadecadienoate. In the 9,12–12,15–mixture, it is not possible to assess what contribution the 12,15–isomer had on lowering the level of 20:3ω9, because the large amount of methyl linoleate in the mixture will, by itself, suppress the level of 20:3ω9.

In a previous study, the eicosatetraenoic acid in rats maintained on a fat-deficient diet was shown to be a mixture of 20:4ω6 and 20:4ω7 (13). If 7,13–eicosadienoate is converted to 20:3ω7 by desaturation at position 10, an increase in both 20:3ω7 and 20:4ω7 should be expected. Under the conditions of GLC used in this study it is unlikely that 20:3ω7 would be resolved from 20:3ω9. However, failure to observe an increase in the level of eicosatrienoate, or 20.4 compared with the fat-deficient controls, suggests that little, if any, of the 7,13–eicosadienoate was converted to 20:3ω7 or to 20:4ω7. The possibility remains, however, that this transformation occurs but that the resulting 20:3ω7 and 20:4ω7 have such a rapid turnover that they do not accumulate to any significant extent in tissue lipids. If this is true, an unequivocal answer concerning the extent of conversion could be obtained only through the use of 7,13–eicosadienoate labeled with either ^{14}C or tritium.

If the three experimental dienoic acids are metabolized according to the third hypothesis, unusual fatty acids having GLC retention times other than those of acids found in the fat-deficient control animals should be observed. Failure to detect any PUFA with a retention time different from the fat-deficient controls indicates that this proposed metabolic pathway is unlikely.

Several investigations have been reported in which attempts have been made to establish what type of fatty acid can serve as a precursor for PUFA. In a detailed study Klenk (21) reported that the isomer of linoleate, 10,13–octadecadienoate (18:2ω5), was not converted to longer chain PUFA's. Likewise the isomer of linolenate, 8,11,14–octadecatrienoate (18:3ω4), was not converted to any significant extent to longer chain PUFA. Ex-

periments at The Hormel Institute have shown that 17:2ω6 and 19:2ω6, in which the double bond system at the methyl end of the molecule is the same as for linoleate, but in which the proximal end is odd, are not elongated (2,22). Schlenk et al. (23) have shown, however, that the odd-chain acids, 9,12-haptadecadienoate (17:2ω5) and 6,9,12–heptadecatrienoate, (17:3ω5) are converted to longer chain PUFA. These two acids resemble linoleate and γ-linolenate in that the double bond system is located the same distance from the carboxyl group. The amount of longer odd-chain PUFA formed when these acids were fed to fat-deficient rats corresponded closely with the increase in longer chain ω6 acids when linoleate and γ-linoleate were the dietary variables.

Klenk (21) has shown that both 16:3ω3 and 16:4ω3 enter the ω3 metabolic pathway. The isomeric compound 16:4ω1 is, however, metabolically inactive. According to Reiser et al. (24) 8:1ω6 is converted to linoleate by the chicken. The same conversion does not occur in the rat (25).

In the studies described here, it is somewhat surprising that 12,15–octadecadienoate was not converted to linolenate. The desaturation at position 9 might be expected since stearate and palmitate are converted respectively to oleate and palmitoleate by desaturation at position 9. This observation is even more puzzling in view of the recent report by Takagi (26) that the rat is able to introduce a double bond into position 8 of 5,11,14–eicosatrienoate to yield arachidonate.

Incorporation of Fatty Acids Into Lecithin

The enzyme systems which incorporate fatty acids into lecithin exhibit a high degree of specificity, for the β position of lecithin contains predominantly unsaturated acids, whereas the α position contains primarily saturated acids (27,28). In view of the low level of incorporation of the experimental dienoic acids into tissue lipids, it was of interest to establish whether these components were incorporated into lecithin and, if they are incorporated, whether they were in the α or β position.

The fatty acid compositions of lecithin, lysolecithin and the fatty acid which were liberated from the β position of those animals fed 9,15-octadecadienoate, the 9,12–12,15–octadienoate mixture or a fat-free diet are presented in Table II. The distribution of fatty acids on the lecithin isolated from the fat-

TABLE II

Positional Distribution of Fatty Acids in Liver Lecithin
(Area Percentage of GLC)

Fatty acid	Untreated lecithin	Lysolecithin	Liberated fatty acid	Distribution on Lecithin	
				α	β
A. Rats on fat-free control diet					
16:0	22.3	35.0	8.7	80	20
16:1	6.2	4.9	6.1	45	55
18:0	22.2	43.8	1.8	96	4
18:1	27.1	14.9	39.8	27	73
9,12–18:2	*1.3*	*0.4*	*1.8*	*17*	*83*
20:3ω9	17.7	1.2	35.2	3	97
20:3ω6	Trace	—	—	—	—
20:4	3.3	Trace	6.6	0	100
B. Rats fed 9,15–18:2					
16:0	31.2	55.0	3.2	81	19
16:1	10.4	5.4	17.5	24	76
18:0	11.0	28.6	0.8	97	3
18:1	30.1	10.1	50.2	17	83
9,12–18:2	*2.1*	*0.4*	*3.2*	*10*	*90*
9,15–18:2	*1.5*	*0.4*	*3.1*	*12*	*88*
20:3ω9	11.6	0.5	18.8	3	97
20:3ω6	Trace	—	—	—	—
20:4	2.3	Trace	3.4	0	100
C. Rats fed 9,12 and 12,15–18:2					
16:0	22.7	43.2	4.3	90	10
16:1	4.3	4.8	8.0	38	62
18:0	20.0	35.2	1.5	96	4
18:1	17.4	9.7	28.7	26	74
9,12–18:2	*11.6*	*2.6*	*24.0*	*10*	*90*
12,15–18:2	*1.6*	*2.0*	*1.6*	*56*	*44*
20:3ω9	5.0	0.8	6.7	10	90
20:3ω6	2.4	0.1	4.9	3	97
20:4	14.9	1.6	20.4	8	92

deficient animals is similar to that reported by Johnson and Ito (29). The saturated acids are esterified primarily to the α position, whereas the unsaturates are located in the β position. The 20:3ω9 behaves as a typical unsaturated acid in that 97% of it is β. Although the fatty acid compositions of the three different lecithin samples differ somewhat, the distribution of any given component is approximately the same for the three. The only significant exception is palmitoleate; the amount of this component esterified to the α position varied from 24 to 45%.

In those animals receiving 9,15-octadecadienoate, the amount incorporated into lecithin represents about 1.5% of the total lecithin fatty acids. The acid is distributed on the α and β positions in a manner similar to the linoleate remaining in the fat-deficient control animals. Conversely, the distributional pattern of 12,15–octadecadienoate differs markedly from either linoleate or 9,15–octadecadienoate, whereas about 90% of the 9,12– or 9,15–octadecadienoate is located in the β position, only 44% of the 12,15–isomer is esterified to the β position. The difference observed in the incorporations of 9,15– and 12,15–octadecadienoate probably is due to the location of the double bond system within the molecule. In the 9,15–isomer the double bonds are widely separated in contrast to the double bonds in the 12,15–isomer, which are localized near the methyl end of the molecule. The proximal end of the latter molecule, i.e., the first 12 carbon atoms, imparts a higher degree of those properties characteristic of saturated fatty acids and causes the 12,15–isomer to be distributed in liver lecithin as if it were a hybrid of saturated and unsaturated acids.

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Pentacyclic Triterpenes of *Jurinea anatolica* Boiss. and *Jurinea consanguinea* DC. Fruit

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ABSTRACT

Pentacyclic triterpene alcohols, together with their acetate, myristate, and palmitate esters, comprise about 40% of the petroleum ether extract of the fruit (seed and pericarp) of two *Jurinea* species. All the triterpene esters and a portion of the free triterpene alcohols in the *J. anatolica* extract are derived from the pericarp portion of the fruit. The triterpene alcohol moieties and their approximate percentage of the hydrolyzed extract of *J. anatolica* fruit are α-amyrin, 5%; β-amyrin, 6%; lupeol, 6%; and ψ-taraxasterol plus taraxasterol, 16%. *J. consanguinea* fruit extract yields essentially the same amount of the same five triterpene alcohols on hydrolysis. These concentrations of triterpenoid materials are believed to be the largest found in plant tissues.

INTRODUCTION

JURINEA PLANTS are biennials or perennials of the Compositae native to central and southern Europe and central Asia. The plants apparently are not cultivated and no description of their chemical constituents has been found. *Jurinea* fruits (seed and pericarp) were analyzed in our laboratory as part of a screening program directed toward findings new plant sources of useful chemical materials.

Preliminary results indicated that the oils contained a large amount of material that was not the normally expected triglyceride. We have shown that this material is comprised of the triterpenes α-amyrin, β-amyrin, lupeol, taraxasterol, and ψ-taraxasterol primarily in the form of their acetate, myristate, and palmitate esters (Scheme I). Smaller amounts of free triterpene alcohols are also present. The glyceride portion of the *Jurinea* fruit extract contained crepenynic acid (1) in addition to the acids which are common to most seed lipids.

Pentacyclic triterpenes are widely distributed in plant materials (2–4), especially the five triterpene alcohols we describe. The most frequent sources appear to be plant tissues other than seeds. However, recent examples show that three of the five triterpene alcohols [lupeol (5) and α- and β-amyrin (6,7)] have been isolated from fruit extracts. While not generally as widespread as lupeol or the amyrins, taraxasterol and ψ-taraxasterol occur quite frequently (8–10) but not exclusively (11) in Compositae plants. Lupeol and β-amyrin are also found in Compositae plants [cf. K. V. Rao (12)], but to our knowledge no single source was previously shown to yield all five of these alcohols. The richest sources of triterpene esters previously described are a crude latex containing 15% of lupeol esters (13) and another latex containing 44% of triterpene acetates (14). The quantity of triterpenoid material usually found in plant tissue, however, is small by comparison with the large amounts of triterpene alcohols we have isolated from the petroleum ether extracts of *Jurinea* fruit.

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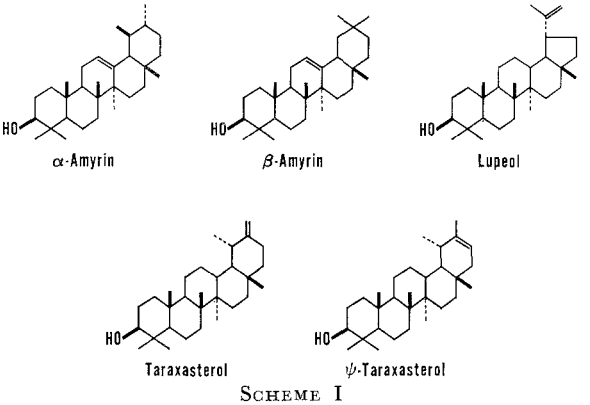

SCHEME I

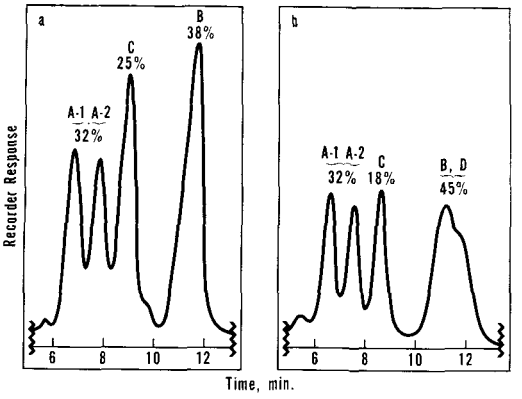

FIG. 1. GLC of *J. anatolica* triterpene acetates prepared (a) from acid-hydrolyzed oil; (b) from alkali-hydrolyzed oil (See Materials and Methods for analytical details). Peaks are identified by letters corresponding to the TLC spots shown in Fig. 2, samples 1 and 3. Key to peaks: *A-1*, β-amyrin; *A-2*, α-amyrin; *B*, ψ-taraxasterol; *C*, lupeol; and *D*, taraxasterol.

PROCEDURES AND DATA

Materials and Methods

Melting points were determined with a Fisher-Johns block and are uncorrected. Infrared (IR) spectra were obtained from 1% solutions in CS_2 or CCl_4 with a Perkin-Elmer Infracord, model 137. Nuclear magnetic resonance (NMR) spectra were determined with a Varian A-60 spectrometer with deuterochloroform as solvent and tetramethylsilane as the internal reference. α-Amyrin and β-amyrin were purchased from K & K Chemical Co.

Transesterification reactions were carried out in 1% H_2SO_4 in MeOH (v/v) at reflux for 3 or 4 hr. The solution was diluted with water and the products were extracted with diethyl ether.

Saponifications were done with 4 or 5% KOH in MeOH at reflux for 2–4 hr. The solution was diluted with 2 volumes of water and extracted thoroughly (6 times) with redistilled hexane. In some saponifications the rather extended reflux periods were necessary to effect complete solution of the sample.

The acetylations were achieved by allowing the alcohols to stand overnight at room temperature in a solution of pyridine:acetic anhydride (1:1). Crushed ice was added to the reaction mixture until heat evolution ceased. Then the mixture was diluted further with water and the products were extracted with diethyl ether. The extracts from all three of these reactions were washed with water before the solvent was removed.

Analysis of the extracted oil by gas-liquid chromatography (GLC) was done on a 19.75 $\times$ 0.125 in. O.D. stainless steel column packed with 3% JXR (15) on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc.). The analysis was begun at 200C and the column temperature was programmed at 4C/min. Helium was the carrier gas and a hydrogen flame ionization detector was used. All analyses of the triterpene acetates (Fig. 1) were done on a 39.50 $\times$ 0.125 in. O.D. stainless steel column packed with 10% phosphoric acid-stabilized DEGS (diethylene glycol succinate polyester) on 120–140 mesh Chromsorb W (Analabs). This column was run isothermally at 233C with helium as the carrier gas. A hydrogen flame ionization detector was used. Samples were injected as 10% solutions in $CHCl_3$, and the resulting peaks were identified by comparison of their retention characteristics with those of known compounds. These two columns were used in an F & M Model 810 Research Chromatograph.

All methyl ester samples as well as acetic acid were analyzed with a Burrell Kromo-Tog K-5 (thermal conductivity detector) instrument equipped with a 6 ft. $\times$ 0.25 in. I.D. glass column packed with 20% LAC-2 R-446 polyester on 60–80 mesh celite. The analyses were carried out isothermally at 195C with helium as the carrier gas. All reported percentages from GLC are area percentages.

Routine analyses of the triterpene acetates by thin-layer chromatography (TLC) were done on 250 μ layers of Silica Gel G impregnated with 20% $AgNO_3$. The solvent was benzene:hexane (40:60) and the spots were visualized by charring at 110C after the plate had been sprayed with a 10% solution of CrO_3 in aqueous 50% H_2SO_4. TLC analyses of the crude extracts were done on plain Silica Gel G using ether:petroleum ether (10–90). The spots were visualized in the same manner as those on $AgNO_3$ plates.

Preparative TLC separations were accomplished similarly except the layers were 1 mm thick and the sample bands were located by viewing the plates under ultraviolet light after they had been sprayed with an ethanolic solution of 2,7–dichlorofluorescein. The portions of the layer containing sample were marked, removed from the plate with a glass vacuum apparatus, and extracted repeatedly with benzene. The maximum loading that could be used and still have adequate separation was 20 μl of a 5% solution (1 mg) of the sample in $CHCl_3$ per spot. With this technique, each plate had a capacity of about 40 mg of sample applied as 40 separate spots.

Preparation and Analysis of Jurinea anatolica Oil

Ground *J. anatolica* Boiss. fruit (38 g) was extracted overnight with petroleum ether (bp 30-60C) in a Soxhlet apparatus; yield of oil, 7.7 g or 20.2%. The IR spectrum, 1% in CCl_4, showed a strong absorption maximum at 1230 cm^{-1} (–OAc). GLC of the oil indicated that 76% of the triterpene esters were acetates, 16% were palmitates, and 8% were myristates. These values should be considered approximate because detector response variations for these esters were not known.

A portion of the oil (2.0 g) was chromatographed on a Silica Gel G column (1.4 × 30 cm) with benzene. Fractions of 5 ml each were collected and monitored by TLC. Pure triterpene esters were the first to come off the column, followed by the bulk of the sample, which was still a mixture. The last fractions contained only triglycerides. The triterpene ester fraction and the triglyceride fraction each amounted to about 5% of the weight of oil put on the column. The triterpene ester fraction was saponified and the alkaline solution evaporated to dryness in vacuo. The residue was cooled in an ice bath, covered with a layer of ether, and acidified with concentrated HCl. The acidified mixture was shaken thoroughly and the ether layer removed and analyzed by GLC. Acetic acid was the only constituent detected, but the analysis was not continued long enough to detect acids longer than 10 carbons. However, GLC of the methyl esters resulting from treatment of the remaining ethereal extract with diazomethane, indicated that methyl palmitate and methyl myristate were present in a ratio of 2:1, and that other components in the range C_6 to C_{22} were absent.

Isolation and Analysis of Triterpene Acetate Mixture

A 5.20 g sample of *J. anatolica* oil was transesterified and the products were recovered; yield, 4.95 g. These products (4.88 g) were separated on a 2.1 × 36 cm column of 100-140 mesh Absorbosil CAB (Applied Science Laboratories, Inc.) by eluting with increasing amounts (5% to 40%, v/v) of diethyl ether in petroleum ether (bp 30-60C). Fractions of 10 ml each were collected and analyzed by TLC. The fractions containing normal methyl esters were combined and the combined sample was analyzed by GLC which gave the following composition: myristate, 2%; palmitate, 11%; stearate, 3%; oleate, 21%; linoleate, 44%; and crepenynate, 15%. Those fractions containing only triterpene alcohols were combined; total yield, 1.65 g or 34% of the total ester mixture. Total recovery of material from the column was 99%. The IR spectrum of the triterpene alcohols, 1% in CCl_4, showed maxima at 3630 cm^{-1} (–OH) and 885 cm^{-1} (=CH_2). Acetylation gave the triterpene acetate mixture, which had IR maxima (1%, CS_2) at 1230 cm^{-1} (–OAc) and 885 cm^{-1}. Hydroxyl absorption was absent. GLC analysis of these acetates (Fig. 1a) indicated four major components in the percentages shown and TLC analysis showed three major spots (Fig. 2, sample 1).

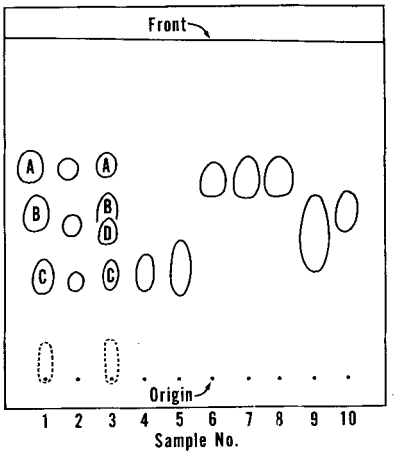

FIG. 2. TLC of triterpene acetates (see Materials and Methods for details). Key to samples: *1*, mixture prepared from acid-hydrolyzed *J. anatolica* oil; *2*, mixture of samples 5, 7, and 10; *3*, mixture prepared from alkali-hydrolyzed *J. anatolica* oil; *4*, lupeol (from *J. anatolica*); *5*, lupeol; *6*, β-amyrin (from *J. anatolica*); *7*, β-amyrin; *8*, α-amyrin; 9, ψ-taraxasterol (from *J. anatolica*); and *10*, ψ-taraxasterol.

Isolation and Identification of Triterpene Acetates A-1, B, and C

Preparative TLC on $AgNO_3$-impregnated Silica Gel G enabled separation of the triterpene acetates into three fractions, A, B, and C. Fraction A (34% of mixture) was crystallized from $CHCl_3$:MeOH (1:4) at –18C. The crystals obtained were recrystallized a number of times to give component A-1, β-amyrin acetate, mp 236-241C, $[\alpha]_D^{25}$ +84° (c = 2.65, $CHCl_3$), and mixed melting point with authentic β-amyrin acetate, 237-241C. Authentic β-amyrin acetate and component A-1 gave superimposable IR and NMR spectra and were identical according to GLC. Fig. 2, samples 6 and 7, shows that the TLC R_f values of component A-1 and β-amyrin acetate are the same. Saponification of A-1 gave β-amyrin, mp and mixed mp with an authentic sample, 198-199C.

After numerous crystalizations from $CHCl_3$:MeOH (1:4) at –18C, fraction B (48%) gave component B (Fig. 1a), mp 238-240C, $[\alpha]_D^{25}$ +56° (c = 5.46, $CHCl_3$), and mixed mp with authentic ψ-taraxasterol acetate 237-239C. Component B and ψ-taraxasterol acetate gave superimposable IR and NMR spectra and identical retention characteristics by GLC. The difference shown on TLC analysis (Fig. 2, samples 9 and 10) is attributable to a difference in the amount of sample spotted on the plate. Saponification of B gave ψ-taraxasterol, mp 220-221C, and mixed melting point with authentic ψ-taraxasterol, 220-221C.

Repeated crystallization of fraction C (18%) from $CHCl_3$:MeOH (1:4) gave component C, mp 219-220C, $[\alpha]_D^{25}$ +44° (c = 2.85, $CHCl_3$), and mixed melting point with authentic lupeol acetate, 219-220C. Component C and lupeol acetate gave superimposable IR and NMR spectra and identical retention characteristics by GLC. The characteristic feature of the IR spectrum (1% in CCl_4) was a strong band at 885 cm^{-1} (=CH_2). The difference indicated by TLC between C and lupeol acetate (Fig. 2, samples 4 and 5) is again caused by different sample loadings. Saponification of component C gave lupeol, mp 217-218C, and mixed melting point with authentic lupeol, 217-218C.

Isolation and Identification of Triterpene Acetate D

A 0.812 g sample of *J. anatolica* oil was saponified and the unsaponifiable portion (41%) was recovered and acetylated. Analysis of the product by TLC (Fig. 2, sample 3) and GLC (Fig. 1b) showed an additional component (D) which did not appear in the triterpene mixture resulting from acid transesterification of *J. anatolica* oil. The resolution of component D from ψ-taraxasterol acetate on preparative TLC was poor, but an acceptably pure specimen was isolated by removing only the lower half of the spot, which had minimal contamination by ψ-taraxasterol acetate. Component D corresponded to the shoulder observed on the GLC peak for ψ-taraxasterol acetate. Its IR spectrum (1% in CCl_4) had a maximum at 879 cm^{-1} (=CH_2), which is slightly different from the corresponding maximum (885 cm^{-1}) in the lupeol acetate spectrum. Numerous crystallizations of D from $CHCl_3$:MeOH (1:4), and finally from ethyl acetate at –18C, gave a product with mp 246-250C [literature value for taraxasterol acetate 251-252C (8)]. Taraxasterol acetate (component D) was refluxed 4 hr with 1% H_2SO_4 in MeOH and the resulting alcohol, mp 217-220C, did not depress the melting point of ψ-taraxasterol and had no =CH_2 absorption in the IR. Reacetylation of this alcohol gave ψ-taraxasterol acetate, identical with that isolated earlier. Saponification of D gave an alcohol that had an IR maximum at 879 cm^{-1} and mp 223-226C, in agreement with the melting point reported for taraxasterol (9). An admixture with lupeol (which also contains a =CH_2 grouping) melted at 175-184C. Unfortunately, an authentic specimen of taraxasterol was not available for direct comparison.

Isolation and Identification of Triterpene Acetate A-2

Fraction A (Fig. 2, sample 3), resulting from the preparative TLC separation of the acetate mixture derived by alkaline hydrolysis of *J. anatolica* oil, was crystallized from a 30% solution in $CHCl_3$:MeOH (1:8). The liquor was concentrated and crystallized again to remove essentially all the β-amyrin acetate (A-1). The residue obtained from the liquor after this second crystallization was dissolved in $CHCl_3$:MeOH (1:4) and recrystallized four times from this solvent. The final product (A-2), mp 220-222C, was identical with α-amyrin acetate according to IR, GLC, TLC, and mixed melting point methods. Saponification of A-2 gave α-amyrin, melting point and mixed melting point with authentic α-amyrin, 180-182C.

Composition of Jurinea anatolica Pericarp and Seed Fractions

J. anatolica fruit was dissected by hand and separated with the aid of a microscope into pericarp (35%) and seed (65%) fractions. Each fraction was ground and was extracted 6 hr in a Butt apparatus with petroleum ether (bp 30-60C). The pericarp extract (21%) was shown by TLC on Silica Gel G to consist almost exclusively of triterpene alcohol esters and free triterpene alcohols. Only a trace of triglyceride material was present. The seed extract contained triglycerides and about 12% free triterpene alcohols but no triterpene esters (by TLC). Analysis of the extracts by IR indicated strong acetate absorption (1230 cm^{-1}) in the pericarp extract, but none in the seed extract.

Composition of Jurinea consanguinea Fruit Extract

Ground *J. consanguinea* DC. fruit was extracted for 6 hr in a Butt apparatus with petroleum ether (bp 30-60C); yield of oil = 20.0%. The IR, TLC, and GLC analyses of this oil gave essentially the same results described for *J. anatolica* oil. Saponification of

J. consanguinea oil and recovery of the products as described above yielded 45% of unsaponifiable material. TLC analysis of the acetylated unsaponifiables gave the same pattern shown in Fig. 2, sample 3. Analysis of this acetate mixture by GLC gave the following results: α-amyrin, 13%; β-amyrin, 18%; lupeol, 24%; and ψ-taraxasterol plus taraxasterol, 43%. Esterification of the fatty acid fraction resulting from saponification of the oil gave a mixture of methyl esters comprised of: myristate, 5%; palmitate, 17%; stearate, 3%; oleate, 24%; linoleate, 38%; linolenate, 0.5%; arachidate, 1%; and crepenynate, 11%. This analysis excludes acetic acid which was volatilized during workup of the ester mixture.

DISCUSSION

The identification of acetic, myristic, and palmitic acids as products from saponification of the triterpene esters (from column chromatography of *J. anatolica* oil) indicates that these are the only acids esterified with the triterpene alcohols. Since acetoglycerides, which are known to occur naturally (16), are not present in this oil (by GLC), all of the acetate absorption observed in the IR spectrum (1230 cm^{-1}) is associated with the triterpene ester portion.

The triterpene alcohol mixture isolated by column chromatography from the acid-catalyzed transesterification products could not be separated by TLC on either Silica Gel G or silver nitrate-impregnated Silica Gel G (17). Some degree of separation was obtained by temperature-programmed GLC analysis but it was not sufficient for identification purposes. After the alcohols were converted to acetates, however, excellent separation of the components was achieved with both silver nitrate TLC (Fig. 2, sample 1) and GLC (Fig. 1a). The identification of peaks shown in Fig. 1a and 1b is based on analyses of the known acetates individually and as a mixture. Similarly, the thre spots appearing in Fig. 2, sample 1 correspond to spots obtained by analysis of the individual known acetates and of a mixture of the known compounds.

Preparative silver nitrate TLC was the method chosen to isolate the individual triterpene acetates because we felt the degree of separation observed on the TLC plates would not offset the loss of resolution one might expect when changing to a column chromatographic method. This would result in lower yields and less pure fractions due to overlap. GLC analyses of the fractions obtained by preparative TLC (fractions A, B, and C, corresponding to the spots shown in Fig. 2, sample 1) indicated they were quite pure. Fraction A contained both α- and β-amyrin acetates as expected but no other impurities.

Fraction A accounted for 34% of the acetate mixture; fraction B, 48%; and fraction C, 18%. The discrepancy between these values and those shown in Fig. 1a is caused by a minor component of the mixture. The shoulder on GLC peak C is included in the 25% indicated, but on TLC this minor component migrates with component B and therefore is included in the weight percentage of component B. This behavior accounts for the 10% difference between the GLC and TLC values for component B. Other minor components in the mixture total 5%.

Components A-1, B, and C were quite readily obtained in pure form by the crystallization procedures already described. NMR (18,19) and IR spectra were obtained for each of these acetates and they were identical with those of pure samples of acetates of β-amyrin, ψ-taraxasterol, and lupeol (A-1, B, and C, respectively). All other analytical data from analysis of these three components agreed with data resulting from analysis of the corresponding known compounds. Alkaline hydrolysis of A-1, B, and C to free triterpene alcohols and comparison of these alcohols with known materials provided further proof of structure. The identity of component A-2, which we could not purify by crystallization of fraction A, will be discussed below.

The triterpene alcohols from alkaline hydrolysis of *J. anatolica* oil were converted to acetates by the usual method and were analyzed by TLC and GLC. Four of the triterpene acetates in this mixture were the same as those in the acid-hydrolysis preparation, but an additional component (D) was detected by TLC (Fig. 2, sample 3) and confirmed by GLC data shown in Fig. 1b.

Component D was isolated by preparative silver nitrate TLC, and strong terminal methylene absorption was the distinguishing feature of its IR spectrum. Though extremely difficult to purify without excessive losses, a product was obtained which was identified as taraxasterol acetate. The acid-catalyzed rearrangement of taraxasterol to ψ-taraxasterol is well documented (20,21), although the reaction usually has been carried out under slightly different conditions than reported here. It is not surprising, therefore, that taraxasterol was not detected in the triterpene mixture isolated by acid hydrolysis (Fig. 1a) of the oil. However,

TABLE I
Physical Constants of Triterpene Alcohols and Acetates

Compound	From *Jurinea anatolica* fruit extract			Literature-values	
	mp, C	mixed mp, C	$[\alpha]_D^{25}$, $CHCl_3$	mp, C	$[\alpha]_D$
α-Amyrin	180-182	180-182	—	182-184	+80° (6)
α-Amyrin acetate	220-222	220-222	—	220-222	+82° (6)
β-Amyrin	198-199	198-199	—	199-200	+88° (3)
β-Amyrin acetate	236-241	237-241	+84°	240-242	+90° (6)
Lupeol	217-218	217-218	—	216-217	+33°(13)
Lupeol acetate	219-220	219-220	+44°	220	+47° (3)
Ψ-Taraxasterol	220-221	220-221	—	220-221	+50° (3)
Ψ-Taraxasterol acetate	238-240	237-239	+56°	240-241	+53° (3)
Taraxasterol	223-226	—	—	222-225	+93° (9)
Taraxasterol acetate	246-250	—	—	251-252	+100° (8)

Fig. 1b shows that ψ-taraxasterol (in addition to taraxasterol) is present in the alkaline hydrolysis mixture. This indicates that a substantial portion of the ψ-taraxasterol isolated from the acid hydrolysis mixture was actually present in the original extract and was not all derived by acid rearrangement of taraxasterol. Both of these alcohols are stable to alkali.

We were successful in isolating component A-2 from fraction A of the triterpene acetate mixture derived via alkaline hydrolysis of the oil, whereas we could not isolate it pure from the corresponding acid hydrolysis preparation. GLC, TLC, and mp data prove component A-2 is α-amyrin acetate. NMR spectra and optical rotations were not obtained for α-amyrin acetate and taraxasterol acetate because of insufficient pure material.

The physical constants of the triterpene materials isolated from *J. anatolica* fruit extract are shown in Table I along with selected literature values.

The fruit extract of another species, *J. consanguinea*, also contained a large amount of triterpenes which were isolated via the alkaline hydrolysis method and acetylated by the usual procedure. TLC analysis of these acetates on silver nitrate-impregnated Silica Gel G gave a pattern the same as that shown in Fig. 2, sample 3, and GLC analysis of this acetate mixture indicated that the same five triterpenes in *J. anatolica* oil were also in this mixture with only minor variations in percentages. Analysis of the fatty acid methyl esters from *J. consanguinea* oil by GLC gave essentially the same composition as did those from *J. anatolica* oil. The fruit extract composition for these two species was so similar that no isolation of individual components was done on the *J. consanguinea* extract.

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Autoxidation of Tissue Lipids. I. Incorporation of Dietary Fatty Acids and Formation of Monocarbonyl Compounds in Adipose Tissue Lipids of the Vitamin E-Deficient Rat

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ABSTRACT

Male weanling rats were fed vitamin E-deficient and vitamin E-supplemented diets containing 5% corn oil or cod-liver oil for 16 weeks, after which their adipose tissue lipids were extracted and analyzed in a nitrogen atmosphere for carbonyl compounds and fatty acids.

The vitamin E-deficient cod-liver oil-fed rats, exhibiting incisor depigmentation and darkened adipose tissue, yielded lipids which had a lower iodine value, contained less polyunsaturated fatty acids, and contained more carbonyl compounds, particularly alkanals and alk-2-enals, than the lipids from the animals fed the vitamin E-supplemented cod-liver oil diet. The tissues of the vitamin E-deficient corn oil-fed rats contained less linoleate and more monocarbonyl compounds than those of the vitamin E-supplemented corn oil-fed animals.

The results indicate that vitamin E protection is necessary for the incorporation of C_{20} and C_{22} fatty acids into the tissues from the diet and that in the deficiency of vitamin E, a low level of autoxidation occurs in the tissues.

INTRODUCTION

THE TERM "peroxidation" has largely replaced "autoxidation" in describing the phenomenon that occurs in vitamin E-deficient animal tissues, because of the empirical nature of the analyses on which the description is based. The principal source of evidence, the 2-thiobarbituric acid (TBA) test, apparently detects a number of unknown compounds that are undoubtedly related in some way to classical autoxidation (1). The expression of the results of the test in terms of malonaldehyde is only a matter of convenience since other substances can be responsible (1) and since malonaldehyde could not be isolated from autoxidized methyl linoleate that had a significant TBA value (2,3). Other studies indicate that autoxidized methyl linoleate should not even exhibit a TBA value (4). In spite of these criticisms, it is evident that the TBA test is of great value in the assessment of the vitamin E status of tissues.

The second source of evidence, the peroxide determination, is only somewhat less empirical with respect to autoxidation and several studies have recently shown that if air is carefully excluded from the reaction, peroxides are not found in vitamin E-deficient tissues (5,6).

Since monocarbonyl compounds have been shown to be reliable secondary products of the autoxidation of lipids and their component fatty acids, and since the carbonyl patterns are specific for the fatty acids involved (7-10), this experiment was designed to isolate any carbonyl compounds that may be present in the tissues of vitamin E-deficient animals and to observe the pattern of these compounds in comparison with the patterns which arise during the autoxidation of other fats and fatty acids.

EXPERIMENTAL

Treatment of Animals

Both corn oil and cod-liver oil were used in this study because of the simple composition of the former and the complex composition of the latter. Cod-liver oil produces classical vitamin E deficiency symptoms rapidly (11) and contains major quantities of polyunsaturated fatty acids of the linolenate family not normally found in tissues and which should produce a typical carbonyl pattern if incorporated into the tissues and oxidized.

Four groups of six male weanling rats (Charles River strain) were fed purified vitamin E-deficient and -supplemented diets containing 5% stripped corn oil (Distillation Products Industries) or cod-liver oil (General Biochemicals Corp.) for 16 weeks. The diets consisted of 20% vitamin-free test casein, 4% Wesson-modified salt mixture, 5% vitamin B complex mixture (all from General Biochemicals Corp.), and 60% sucrose. All diets were supplemented with 0.4 mg vitamin K per 100 g of diet; the corn oil diets were supplemented with 22.4 U.S.P. units of Vitamin A (palmitate) and 2.1 U.S.P. units of Vitamin D per g of diet to

simulate the vitamin A and D status of the cod-liver oil; and the control diets were supplemented with 200 mg D,L-α-tocopherol acetate per kg of diet. The diets were prepared every four days and feedings were daily in ad lib. quantities with refrigerator storage between feedings. The oils were stored in the freezer under nitrogen between preparations.

After 16 weeks, the average weight of the vitamin E-deficient cod-liver oil fed animals was 257 g. These animals showed incisor depigmentation and darkened adipose tissue. The average weight of the other three groups was between 402 and 439 g and no vitamin E deficiency symptoms were evident.

Lipid Analysis

Isolation of Lipids. After 16 weeks, the animals were chloroformed and placed in a polyethylene glove bag (Instruments for Research & Industry, Cheltenham, Pa.) under positive nitrogen pressure. All solvents had been purged with nitrogen for 5 min before introduction into the glove bag with the other equipment. The perirenal and epididymal fat pads were removed rapidly, weighed, and extracted in a blender with chloroform-methanol (12). The extract was dried on a rotary film evaporator (Buchler Instruments, Fort Lee, N. J.) under aspirator vacuum at 40C without exposure to the atmosphere. The evaporator was vented with nitrogen and the dried extract returned to the glove bag and weighed before a 2 mg sample was taken for gas liquid chromatography (GLC) and the remainder subjected to carbonyl analysis.

Fatty Acid Analysis. Methanolysis was accomplished by the recently recommended AOCS procedure (13) followed by immediate GLC using a dual column instrument equipped with a differential hydrogen flame ionization detector. Two 6-ft, 1/8 in. stainless steel columns were packed with 8% polybutandiol succinate (BDS) on 80-100 mesh hexamethyldisilazane treated (HMDS) Chromosorb W. Analyses were obtained with a flow rate of 18 ml per min at STP, injector at 275C, detector at 225C, and columns at 180C or 210C. Sample size was 1 μl of a 10% hexane solution. The chromatograms were recorded on a 10-in. recorder at 30 in. per hour.

The methyl esters were identified by their stearate retention time ratios with reference to those of standards (Hormel Foundation and NIH) and values from the literature (14,15). Fatty acid compositions were calculated as per cent of total methyl esters by triangulation. Quantitative results with the Hormel Foundation's standard methyl ester mixtures No. 1, 2, 5, and 9 agreed with the stated composition data with relative errors of less than 5%. The results of the analyses are presented in Table I.

Monocarbonyl Analysis. The extracted lipid was dissolved in 800 ml of hexane and allowed to flow through a 10 g celite/H_3PO_4/2,4-dinitrophenylhydrazine reaction column (17,18) causing the quantitative conversion of carbonyl compounds to derivatives. After this point further isolation from the atmosphere was not necessary and the eluate from the column was removed from the glove bag. Total carbonyls were then estimated by measuring the absorbance of this solution at a wavelength of 340 mμ with a Beckman DU-2 spectrophotometer using a molar absorptivity of 22,500 (19). The monocarbonyl derivatives were isolated by chromatography on activated magnesia and hydrated alumina (17) and separated into classes on magnesia (20). The classes were then separated into their individual components by means of liquid-liquid partition chromatography between hexane and acetonitrile (21). This step was accomplished with the aid of an ultraviolet flow analysis system consisting of a Bausch & Lomb 505 spectrophotometer equipped with a time-rate accessory and a Sargent SRL recorder, and an LKB automatic fraction collector equipped with an event marker. In this way, absorbance at the wavelength of maximum absorption for the class was recorded against time with the passage of each fraction recorded by the event marker. The chromatographic columns were attached to the system by means of polyethylene tubing and a 1 cm silica flow-through cuvette. Five milliliter fractions were collected.

The identifications of the monocarbonyl derivatives were made on the basis of partition chromatographic peak volumes, class separation chromatographic mobilities, and ultraviolet spectra. Only the alkanal and alk-2-enal fractions from the class separation were partitioned. Quantitative data were obtained spectrophotometrically using the Beckman DU-2 on the pooled fractions comprising a chromatographic peak. Molar absorptivities of 22,500 and 27,500 were used for the alkanal and alk-2-enal derivatives respectively (19). The results are in Table II. After carbonyl removal, the iodine values of the lipids were obtained by the AOCS method (22) and are included in Table I.

DISCUSSION

Fatty Acid Compositions

The influence of vitamin E deficiency on the fatty acid composition of the adipose tissue

TABLE I

Fatty Acid Compositions of Dietary Fats and Tissue Lipids[a] (Weight per cent total methyl esters ± SE)[b]

Fatty acid[c]	Dietary fats		Tissue lipids from rats fed diets containing:				
	Corn oil	Cod liver oil	Corn oil + vit. E	Corn oil	Cod-liver oil + vit. E	Cod-liver oil	Commercial ration
14:0	trace	4.01 ± 0.04	1.81 ± 0.14	2.03 ± 0.06	3.44 ± 0.18	2.97 ± 0.43	2.81 ± 0.12
16:0	12.7 ± 0.40	13.8 ± 0.17	24.3 ± 0.58	25.9 ± 0.76	28.0 ± 1.00	28.4 ± 0.93	31.1 ± 1.10
16:1		10.5 ± 0.10	8.68 ± 0.09	9.87 ± 0.48	13.6 ± 0.28	14.2 ± 0.55	6.58 ± 0.20
16:2		2.55 ± 0.14	0.39 ± 0.06	0.45 ± 0.04	0.91 ± 0.13	0.68 ± 0.04	
18:0	2.54 ± 0.16	2.96 ± 0.05	2.86 ± 0.06	2.40 ± 0.08	2.77 ± 0.30	2.19 ± 0.21	6.75 ± 0.30
18:1	29.2 ± 0.50	27.6 ± 0.57	38.4 ± 0.18	38.8 ± 1.93	38.8[d] ± 1.48	42.4[d] ± 0.67	38.0 ± 0.65
18:2 ω 6	54.9 ± 0.76	1.28 ± 0.05	21.6[e] ± 0.22	19.5[e] ± 0.68	1.70[d] ± 0.13	1.24[d] ± 0.10	14.8 ± 0.32
18:4 ω 3		2.13 ± 0.17			0.52[d] ± 0.05	0.26[d] ± 0.02	
20:1		6.94 ± 0.26	trace	trace	2.98[d] ± 0.09	2.35[d] ± 0.20	
20:5 ω 3		11.5 ± 0.44			1.52[d] ± 0.06	0.70[d] ± 0.07	
22:1		3.70 ± 0.13			trace	trace	
22:5 ω 3		1.11 ± 0.04			0.95[d] ± 0.06	0.50[d] ± 0.05	
22:6 ω 3		8.73 ± 0.30			2.17[d] ± 0.10	1.22[d] ± 0.12	
Iodine Value	122	162	80.8	80.2	71.8	68.0	68.6

[a] Major components, > 1% of one of the fats.

[b] Average of six analyses.

[c] Chain length, number of double bonds. Number after ω indicates position of double bond closest to terminal methyl group.

[d] Difference between control and experimental groups significant at 0.001 probability level (16).

[e] Difference between control and experimental groups significant at 0.01 probability level (16).

of rabbits has been shown to be minor or nonexistent (23,24), whereas it has been suggested that the conversion of 20:4 ω 6 to 22:5 ω 6 is inhibited in the testis of the vitamin E-deficient rat (25). It is interesting to compare the reported increase in arachidonate in the liver lipids of rats (29,30) with the reported decrease in docosahexaenoate in the muscle phospholipids of the same animal (31) under conditions of vitamin E deficiency.

A glance at Table I will indicate the extent of the incorporation of the dietary fatty acids into the tissue lipids. There was, however, an obvious decrease in the polyunsaturated fatty acids of the vitamin E-deficient as compared with the vitamin E-supplemented groups. The iodine values of the lipids from the cod-liver oil-fed animals reflect this decrease which is accompanied by an increase in oleate. In view of the increase in oleate, it can be concluded that since the C_{20} and C_{22} fatty acids arose from the diet, the decrease in eicosaenoate, which is much less susceptible to autoxidation than the polyunsaturated fatty acids, indicates that in the deficiency of vitamin E, there was less efficient incorporation of the C_{20} and C_{22} fatty acids into the tissues from the diet. Had the decrease resulted from in vivo autoxidation, the monoene would not have been affected as is the case when cod-liver oil is allowed to autoxidize in vitro to a high peroxide value (26).

Carbonyl Pattern

Table II reveals that the total carbonyl values are approximately equal for both corn oil-fed groups and the cod-liver oil-fed control, but somewhat higher for the vitamin E-deficient cod-liver oil-fed group. The alkanals and the alk-2-enals were the only classes that were further fractionated. There were no alk-2,4-

TABLE II

Concentrations of Carbonyl Compounds Isolated from Tissue Lipids

Compound	Concentration (moles × 10^{10} per g lipid) from rats fed diets containing:			
	Corn oil + vit. E (95.5 g)[a]	Corn oil (72.5 g)[a]	Cod-liver oil + vit. E (89.4 g)[a]	Cod-liver oil (28.3 g)[a]
Alkanals				
C_{10}	5.05	11.7		
C_9			5.00	46.9
C_8	2.01	6.50		
C_7	4.67	6.52		60.8
C_6	11.8	6.07	18.7	
C_5	81.5	56.3	22.1	
C_4			83.4	849
C_3	12.3			
C_2				35.2
Total	105	99.9	129	992
Alk-2-enals				
C_9			12.3	30.5
C_7			1.90	
C_6				14.7
C_5				38.9
Total			14.2	84.1
Total Carbonyls	22000	21000	20400	25800

[a] Quantity of fat analyzed.

dienals present in any group. There was 7 to 8 times as much saturated aldehyde and 6 times as much alk-2-enal in the tissues of the vitamin E-deficient cod-liver oil-fed animals than in the tissues of the control animals. The major aldehyde was butanal which showed a tenfold increase. The major alk-2-enal was 2-pentenal. There was no significant difference between the two groups fed corn oil. The alk-2-enals were not found in either of these groups.

Since the C_{20} and C_{22} polyunsaturated fatty acids were incorporated into the tissues from a fish oil diet, they were found to belong to the linolenate family with the double bond closest to the terminal methyl group located on the third carbon (Table I). According to the classical mechanism for the decomposition of hydroperoxides (7-10) the two major autoxidation products from this system should be 2-pentenal, as observed, and propanal. That butanal was the major aldehyde formed in autoxidized cod-liver oil with a low peroxide value (26) as well as in these tissues is enigmatic. Mechanisms are available to explain its formation (27), but they do not explain the absence of propanal. Possibly, in these very early stages of autoxidation the mechanism is atypical. This is currently under investigation with cod-liver oil and pure methyl esters of fatty acids from cod-liver oil.

The absence of alk-2,4-dienals is not unexpected since the autoxidation of polyenoic systems is generally confined in the early stages to the double bond in the methyl terminal part of the molecule (28).

In conclusion, considering the concentrations of the carbonyl compounds present in these tissues, it is not remarkable that peroxides at an equivalent level have escaped detection. The utility of the more sensitive carbonyl analysis in the study of in vivo autoxidation is evident.

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Mechanism of Suckling-Rat Hypercholesteremia. II. Cholesterol Biosynthesis and Cholic Acid Turnover Studies[1,2]

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ABSTRACT

Cholesterol biosynthesis from acetate-2-^{14}C by the livers of suckling rats, which is known to be relatively slow, was increased 2- 3-fold within 24 hours after severing the bile duct. Cholesterol synthesis by sham-operated litter mates showed no change under similar treatment. Mevalonate biosynthesis from acetate-2-^{14}C in vitro by recombined liver microsomal supernatant (105,000 × g) fractions from suckling rats also was only 10% of that of comparable recombined fractions from normal controls (young adult rats which were consuming colony diet). Activity was not improved by combining either the microsomal or supernatant fraction from suckling rat livers with the complementary fraction from normal adult livers. On the other hand, activity was restored to 100% when microsomes from livers of duct-severed suckling rats were combined with the supernatant fraction from normal controls. Likewise, recombined liver fractions prepared from adult rats fed synthetic diets exhibited low activity for mevalonate biosynthesis. Activity was restored by bile duct cannulation, but inhibited when cholic acid was infused into the cannulated animal. Therefore, surgical procedures which interrupt the enterohepatic recirculation of bile components lead to a restoration of cholesterol biosynthesis, and, at least in the adult animal where cannulation studies are practicable, this effect can be reversed readily by bile acid infusion.

A slow rate of fecal excretion of ^{14}C-cholic acid was observed in suckling rats and rats fed synthetic diets, apparently reflecting an efficient enterohepatic recirculation of bile salts. The data suggest that under these dietary conditions bile salt retention either directly or indirectly influences hepatic synthesis of cholesterol.

INTRODUCTION

THE SUCKLING RAT is characteristically hypercholesteremic (1-3), has slightly elevated hepatic cholesterol levels (1,2) and fails to respond to most hypocholesteremic agents (2,3). Rat milk provides only small amounts of cholesterol (1) and its high fat content appears to be the most important factor in maintaining suckling-rat hypercholesteremia (2). In hepatic tissue from suckling rats cholesterol biosysthesis from acetate proceeds at a low rate while synthesis from mevalonate is normal (1). A mechanism is thus provided for minimizing the severity of the hypercholesteremia produced by the high fat diet of the suckling rat. Inhibition may be a result of "feedback" control by the slight increase in hepatic cholesterol levels or another mechanism may be involved. In the present communication a defect in the hepatic synthesis of mevalonate from labeled acetate is demonstrated. Synthesis is shown to be restored by interruption of the enterohepatic recirculation of bile components. An observed slow rate of cholic acid elimination is suggested to play a role, although perhaps indirect, in producing conditions unfavorable for mevalonate synthesis in the suckling and synthetic-diet-fed rat.

EXPERIMENTAL

Materials and General Methods

Compounds were purchased as follows: acetate-2-^{14}C, 20.5 mc/mM (Calbiochem, Los Angeles); cholic acid-24-^{14}C, 1.35 mc/mM (Tracerlab, Waltham, Mass.); cholesterol-U-^{3}H, 1060 mc/mM (Nuclear-Chicago, Chicago); glucose-1-phosphate, DL-mevalonic acid lactone, sodium taurocholate, and cholic acid (Nutritional Biochemical Corporation, Cleveland); nicotinamide adenine dinucleotide phosphate (NADP), coenzyme A(CoA) and adenosine

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TABLE I

Synthesis in Vivo of Hepatic Cholesterol from Acetate-2-^{14}C by Sham-Operated or Duct-Severed Suckling Rats

Exper'nt	Treatment	Number of rats	Cholesterol synthesis[a] Mean	Range
			cpm x 10^{-3}	cpm x 10^{-3}
I	Sham	3	55	15-103
	Duct-severed	2	167	144-189
II	Sham	5	70	22-121
	Duct-severed	4	154	84-280

[a] Total cpm per liver recovered after digitonide purification of cholesterol.

triphosphate (ATP) (Calbiochem, Los Angeles).

Radioactivity was estimated (Packard Tri-Carb) with either dioxane or toluene scintillation fluid (2). Corrections for quenching were made with internal standards when necessary. The radioactivity data are subject to a ±5% counting error.

Rats of the Wistar strain were used in all experiments. The basic composition of the colony diet and semipurified diets has been described in detail previously (2,4). Diet C was the colony diet; Diet G was semipurified, contained 2% fat and no cholesterol; Diet M-47, designed to simulate the composition of rat milk, contained 47% of hydrogenated coconut oil; and Diet M-1, isocaloric with M-47, contained 1% of hydrogenated coconut oil.

Bile Duct Surgery

Bile ducts of suckling rats (35-45 g) were exposed under ether anesthesia and ligated close to the small intestine. The ducts were then severed between the liver and the point of the ligation; bile flowed into the peritoneal cavity. In the sham-operated litter mates the bile duct was exposed but not ligated nor cut. After closure of the incisions, the suckling rats were returned to the dams for 24 hr before assay for synthesis of either mevalonate or cholesterol. Cannulation of the bile duct of the suckling rat, employed in preliminary efforts, presented several technical problems, e.g. immobilization; discontinuous flow; and inadequate nourishment. Therefore, severance of the duct was employed to interrupt normal enterohepatic recirculation.

Bile ducts of adult rats were cannulated to provide exterior drainage and to permit infusions. The duct was exposed and an incision made at the mid-duct to allow insertion of a size P10 polyethylene cannula which served as a drainage tube leading from the liver to the exterior. For infusion studies, a second cannula was inserted into the bile dult afferent to the intestine. Thereafter, animals were maintained in restraining cages with access to food and water.

TABLE II

Synthesis in Vitro of Mevalonate from Acetate-2-^{14}C by Recombined Fractions of Rat Liver

Soluble fractions[a]	Microsomes[b]	Mevalonate synthesis[c]
Series A		mean cpm
Normal adult	Normal adult	10,760
Suckling	Suckling	990
Normal adult	Suckling	1,800
Normal adult	Suckling	1,450
Normal adult	None	1,500
Suckling	Normal adult	1,033
Suckling	None	956
Series B		
Normal adult	Normal adult	6,860
Normal adult	Sham-operated suckling	1,080
Normal adult	Duct-severed suckling	7,540

[a] 105,000 x *g* supernatant, equivalent to 0.5 g of fresh liver from a homogenate of 4 normal adult rat livers or 18 suckling rat livers.

[b] 105,000 x *g* pellet, equivalent to 1.0 g of liver from either normal or suckling rats.

[c] Mean values of duplicates.

Assay for Cholesterol Biosynthesis in Vivo

Suckling rats were injected intraperitoneally with acetate-2-^{14}C (2×10^7 cpm) in 0.15 ml of saline solution and placed in cages equipped to trap $^{14}CO_2$. One hour later the rats were decapitated, livers were removed and extracted with hexane-ethanol (75:10, v/v). Extracts were analyzed for total cholesterol, purified as the digitonide (2). Further purification of cholesterol recovered from the digitonide was performed as follows: addition of carrier cholesterol plus uniformly labeled cholesterol-^{3}H; thin-layer chromatography (TLC) on Silica Gel G with hexane-ether-acetic acid (70:30:1, v/v) as solvent system; acetylation with acetic anhydride-pyridine (2:1, v/v); and subjection of the resulting derivative to TLC on 3% silver nitrate-Silica Gel G with a benzene-ethyl acetate (9:1, v/v) solvent system.

Assay for Mevalonate and Fatty Acid Biosynthesis in Vitro

Livers were removed from decapitated rats, cut into pieces, rinsed in ice-cold 0.25 M sucrose, and passed through a tissue press (Harvard) into 3 volumes of ice-cold medium, pH 7.0, containing 0.05 M potassium phosphate, 0.15 M KCl, 0.25 M sucrose, 7.5 mM $MgCl_2$, 1.5 mM glutathione, and 0.03 M nicotinamide per liter. The suspension was homogenized by 6-8 passes with a motor-

driven, loose-fitting Teflon pestle in a plain glass test tube. The homogenate was centrifuged for 15 min at 800 × *g*, followed by 30 min at 8,000 × *g*. The resulting supernatant fraction was filtered through glass wool to remove floating fat particles, and centrifuged at 105,000 × *g* for 1 hr to obtain the microsomal and soluble fractions. The fat layer was removed from the soluble fraction and the microsomes were suspended in homogenizing medium without sucrose. The procedures for incubation and isolation of mevalonate were similar to those of Fimognari and Rodwell (5). Soluble fraction (equivalent to 0.5 g of fresh liver) and microsomes (equivalent to 1 g) were incubated for 2 hr with 6 μmoles of acetate-2-^{14}C (1.4 × 10^6 cpm/μmole), 4 μmoles ATP, 0.2 μmoles CoA, 9μmoles glucose-1-phosphate, 2 μmoles NADP, 2.5 μmoles glutathione, and 10 μmoles of potassium DL-mevalonate. When microsomes or soluble fractions from various treatments were cross-combined and compared for activity, protein was determined by the method of Lowry et al. (6) to establish that similar amounts of complementary fractions had been used in each incubation flask. Incubations were stopped by heating at 80C for 3 min. Denatured protein was removed by centrifugation and washed once with distilled water. The extracts were treated to transform mevalonate into the lactone (7) and the latter was extracted and isolated by Celite chromatography (5,8). Samples analyzed for isotope incorporation into fatty acids were saponified with 10% methanolic KOH, acidified, and extracted with hexane. Methyl esters were prepared with diazomethane (9) and purified by TLC on silica gel G with hexane-ether-acetic acid (70:30:1, v/v) as solvent system. Corrections for losses during purification were provided by a radioactive internal standard as described for cholesterol purification.

Cholic Acid-24-^{14}C Excretion Studies

Litters of suckling rats, 7 days old, were kept with nursing females in metal cages with corn cob litter. Diet C in suspended feed cups was consumed ad libitum by the dams. Aqueous cholic acid-24-^{14}C (170,000 cpm) was force-fed to each suckling rat with a special syringe. Each day a pup was killed and the alcohol-extractable radioactivity of the entire carcass was obtained by grinding thoroughly in a homogenizer (Virtis) and extraction for 24 hr in a soxhlet apparatus. In one experiment, 5 suckling rats force-fed the labeled cholic acid were kept in a screen-bottomed cage without feed cups. Feces collected from the group were extracted with boiling 80% ethanol for assay of radioactivity. Two lactating females alternated every 6 hr to provide milk. In similar experiments, the appearance of label in the fecal material was used to estimate the rate of cholic acid elimination for rats fed various diets for two months from weaning age.

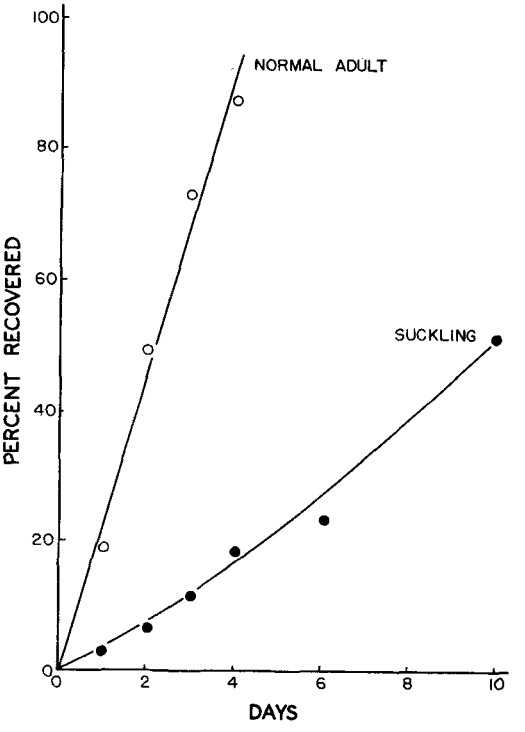

FIG. 1. Fecal excretion of isotope by suckling rats and normal adult rats consuming the colony diet (Diet C) ad libitum. Each was force-fed a single dose of cholic acid-24-^{14}C. The cholic acid-24-^{14}C cochromatographed with authentic cholic acid (TLC: Silica Gel G, ethyl acetate-acetic acid, 24:1 v/v).

RESULTS

In vivo incorporation of acetate-2-^{14}C into hepatic cholesterol of suckling rats 24 hr after severing the bile duct was 2- 3-fold that of sham-operated litter mates (Table I). The cholesterol samples, purified as the digitonides in the first experiment, subsequently lost 5-8% of their activity when further purified by TLC with an internal standard of tritiated cholesterol. It was evident, therefore, that digitonide purification of cholesterol in the present study was adequate and also that the sterol synthesized was indeed cholesterol.

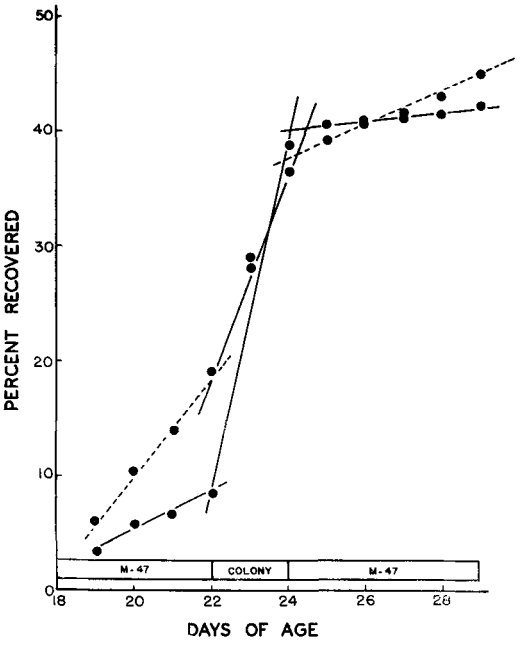

FIG. 2. Effects of dietary changes on fecal excretion of isotope by young rats which received a single dose of cholic acid-24-^{14}C. Suckling rats (broken lines) were weaned and fed colony diet (Diet C) for 2 days, then returned to suckling status. Litter mates (solid line), weaned and fed Diet M-47 which simulated rat's milk, also were shifted to colony diet for the same 2-day period.

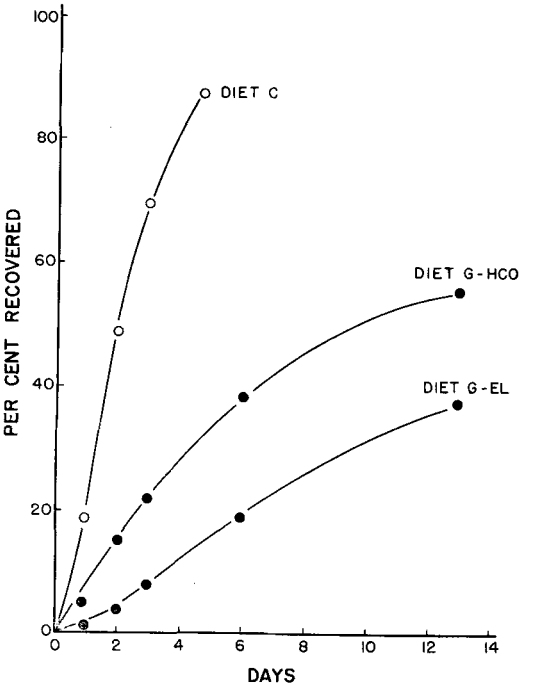

FIG. 3. Effects of continuous feeding of different diets on fecal excretion of isotope by growing rats which received a single dose of cholic acid-24-^{14}C. Diet C was the colony diet; Diet G, a synthetic diet, contained 2% of hydrogenated coconut oil (HCO) or 2% of ethyl linoleate (EL).

Incorporation of acetate-2-^{14}C into mevalonate in vitro by recombined soluble (105,000 × *g* supernatant) plus microsomal fractions from normal rat liver was far superior to that of recombined fractions from suckling rat liver (Table II). Soluble fractions or microsomes from suckling rat liver combined with the complementary fraction from normal liver were not more active than the soluble fractions without microsomes. However, when liver microsomes from duct-severed suckling rats were combined with soluble fraction from normal rats the system incorporated acetate-2-^{14}C into mevalonate satisfactorily (Series B, Table II). Microsomes from sham-operated litter mates combined with soluble fraction from normal rat liver produced relatively little incorporation.

The rate of the cholic acid excretion of the suckling rat was much slower than that of the normal adult rat fed Diet C (Fig. 1). A sharp contrast was also observed in the excretion patterns of cholic acid-24-^{14}C for rats newly weaned with colony diet and for litter mates either still suckling or weaned with Diet M-47 (Fig. 2). Excretion rates, which were relatively slow for 4 days, accelerated in both groups when the animals were changed to Diet C for 2 days; finally, rates retarded again when each was returned to the former diet for 5 days.

Adult rats when fed a semipurified diet (Diet G) also excreted labeled cholic acid slowly in comparison to those fed Diet C (Fig. 3). Liver microsomes from rats fed Diet G when combined with soluble fraction from rats fed Diet C or Diet G did not provide the activity for acetate-2-^{14}C incorporation which was obtained with soluble plus microsomal fractions from normal rats (Table III). As with bile duct severing in the suckling rat, bile duct cannulation of rats fed Diet G significantly improved the ability to incorporate labeled acetate. However, when Diet G rats with bile duct cannulas to the exterior were infused with 10 mg of cholic acid per hour for 24-30 hr through an additional cannula leading to the duodenum, mevalonate synthesis by their recombined liver fractions was markedly depressed (Series B, Table III).

TABLE III

Effect of Diet, Bile Duct Cannulation and Bile Acid Infusion upon the Synthesis of Mevalonate from Acetate-2-^{14}C by Recombined Liver Fractions

Soluble fraction[a]	Microsomes[b]	Mevalonate synthesis[c]
Series A		cpm
Diet C	Diet C	20,500
Diet G	Diet G	1,320
Diet C	Diet G	3,110
Diet C	Diet G, cannula[d]	13,600
Diet G, cannula[d]	Diet G, cannula[d]	6,220
Diet G	Diet C	14,910
Diet G, cannula[d]	Diet C	14,240
Series B		
Diet G, cannula[d]	Diet G, cannula[d]	64,600
Diet G, cannula + infused[e]	Diet G, cannula + infused[e]	2,777

[a] 105.000 x *g* supernatant equivalent to 0.5 g of fresh liver from a homogenate of 2 or more livers from rats fed Diet C or Diet G as indicated.

[b] 105,000 x *g* pellet, equivalent to 1.0 g liver.

[c] Mean values of duplicates.

[d] Bile duct cannulated 24-48 hr before assay.

[e] Bile duct cannulated and duodenum infused at rate of 10 mg of cholic acid per hour for 24-30 hr before assay.

In agreement with the data of Fimognari and Rodwell (5), taurocholate was found to inhibit the biosynthesis of mevalonate from acetate-2-^{14}C in recombined soluble and microsomal fractions from normal rat liver (Table IV). Likewise, a residue from an 80% alcohol extract of a 10,000 $\times$ *g* supernatant from either suckling or normal rat liver reduced acetate-2-^{14}C incorporation into mevalonate (Table IV). Incorporation of mevalonate was inhibited at taurocholate concentrations above 0.25 mM (Fig. 4). Complete inhibition of mevalonate biosynthesis was obtained at taurocholate concentrations which did not impair fatty acid biosynthesis by these recombined liver fractions.

TABLE IV

Inhibiting Effects of Additions upon in Vitro Biosynthesis of Mevalonate

Addition to incubation[a]	% Inhibition[b]
Taurocholate[c], 1 x 10^{-3} M	80
Taurocholate, 2.5 x 10^{-3} M	97
Taurocholate, 5.0 x 10^{-3} M	98
80% Ethanol extract of suckling rat liver[d]	68
80% Ethanol extract of normal rat liver[d]	51

[a] Incubation system consisted of recombined soluble and microsomal fractions from normal rat liver with cofactors and acetate-2-^{14}C as described in the text.

[b] Relative to control flask which received no additions.

[c] Gas chromatography and TLC revealed presence of ca. 5% of free acids, mainly cholic with a trace of deoxycholic.

[d] Dry material equivalent to 0.7 g of liver (net weight).

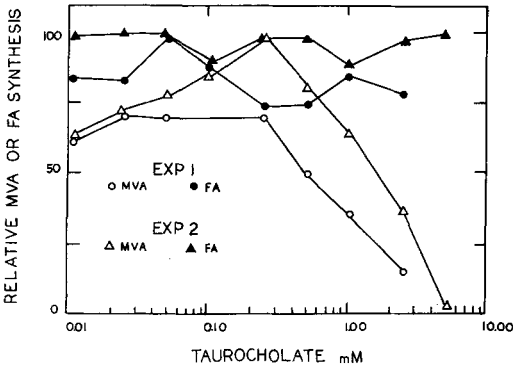

FIG. 4. Effect of taurocholate upon acetate-1-^{14}C incorporation into mevalonate and fatty acids by recombined soluble and microsomal fractions of normal rat liver. MVA, mevalonate; FA, fatty acids.

DISCUSSION

Our observation that mevalonate biosynthesis in vitro with recombined supernatant and microsomes from suckling-rat liver is subnormal is consistent with that of Carroll (1) who showed poor incorporation of acetate, but good incorporation of mevalonate, into cholesterol. The major rate-limiting step in cholesterol biosynthesis in adult rats occurs in the microsomes and the control point is known to precede reactions of mevalonate in experiments involving fasting (10,11), cholesterol feeding (11-14), Triton WR-1339 (10.11), x-irradiation (10,11), and Migicovsky inhibitor (16,17). "Feedback" regulation of the microsomal enzyme, β-hydroxy-β-methylglutaryl reductase, responsible for the final steps in mevalonate formation, is believed to be the principal mechanism involved in inhibition by cholesterol feeding (12-14). In the present experiments reduced mevalonate synthesis by a soluble liver fraction from normal rats when combined with microsomes from suckling rats suggests that a similar feedback mechanism may be responsible for suppressing cholesterol biosynthesis in the suckling rat.

As in our experiments with duct-severed suckling rats and duct-cannulated rats, Myant and Eder (17) found an increase in cholesterol biosynthesis from acetate with liver tissue from adult rats with bile duct cannulas. Such results suggest the presence of an inhibitor of cholesterol biosynthesis in the bile, and its removal from liver by the interruption of the enterohepatic recirculation of a bile constituent. Among the bile constituents, cholesterol is possibly the inhibitor since cholesterol feed-

ing has been shown to result in an inhibition of hepatic synthesis of cholesterol (10-14,18,19). Bloomfield (20) has suggested that the bile acids may be responsible for control of cholesterol biosynthesis through a feedback mechanism of action upon cholesterol formation. Studies by Fimognari and Rodwell (5,21) suggest that bile acids may be responsible for a "feedback" control upon cholesterol biosynthesis by inhibition of the enzyme which results in mevalonate formation, β-hydroxy-β-methylglutaryl reductase. While it has not been established conclusively that bile acids directly inhibit this enzyme in a manner that provides physiological control, our demonstration that ability to synthesize mevalonate can be improved by bile duct cannulation and blocked by bile acid infusion suggests a role for the constituent bile acids in the regulation of hepatic cholesterogenesis. Also, lack of an inhibitory effect upon in vitro fatty acid biosynthesis at taurocholate concentrations which blocked mevalonate biosynthesis (Fig. 4), suggests a degree of specificity for taurocholate, perhaps upon the β-hydroxy-β-methylglutaryl reductase. Experiments show that dietary bile acids inhibit cholesterol biosynthesis consistently (22). Failure to observe an inhibitory effect on cholesterol biosynthesis when bile acids were added to liver slices (23) may reflect a low degree penetration of exogenous bile acid into tissue.

The slow rate of fecal excretion of labeled cholic acid observed in the present study with suckling rats and with rats fed semipurified diets in our experiments as well as Portman's (24) may reflect a highly efficient enterohepatic recirculation of bile salts. This recirculation may provide favorable intestinal conditions for the absorption of cholesterol, and thus maintain serum or hepatic cholesterol levels inhibitory to the mevalonate-synthesizing enzyme system. On the other hand, efficient conservation of bile salts may provide the tissues with levels which we have found inhibitory to cholesterol biosynthesis in vitro. By either mechanism, the increased rate of cholic acid elimination, observed when suckling rats or their litter mates fed Diet M-47 were shifted to colony diet, could lower the feedback stimulus. Such conservation could explain the increase in hepatic cholesterol biosynthesis observed by Carroll (1) when suckling rats were weaned and fed a colony diet and the subnormal rates of biosynthesis of cholesterol in rats fed synthetic diets (25).

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The Isolation and Tentative Identification of Diacylglyceryl Ethers from the Walker 256 Carcinoma of the Rat and a Human Lymphosarcoma[1]

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ABSTRACT

An unusual lipid was observed and subsequently isolated by thin-layer chromatography from the lipids of the Walker 256 carcinoma of the rat and a human lymphosarcoma. This lipid has been tentatively identified as a diacylglyceryl ether by thin-layer chromatography, gas-liquid chromatography and infrared analysis.

INTRODUCTION

LIPIDS OBTAINED from both animal and human tumors in this laboratory (1) were shown to contain an unidentified lipid class by thin-layer chromatography (TLC). The polarity of the unidentified component on thin-layer chromatograms was shown to be identical to the polarity of a glyceryl ether diester such as batyl dimyristate.

This report deals with the methods of isolation and identification of this unusual lipid which was found in the Walker 256 tumor and in a human lymphosarcoma.

MATERIALS AND METHODS

Extraction and Fractionation of Tumor Lipids

Walker 256 carcinoma tissue was obtained from female rats (Carworth Farms-Nelson strain) fed a commercial chow (Purina) diet. Tumor implant material was obtained by macerating tumor tissue from donor rats (Upjohn Company, Kalamazoo, Mich.) in a micro-Waring Blendor. The homogenized cells were diluted in saline solution and immediately injected into the right femoral muscle. Tumors ranging from 15 to 30 g were obtained 10 days after implantation. The animals were anesthetized with ether, killed by exsanguination, and the tumors were removed. Care was taken to remove all the necrotic tissue before the tumor tissue was homogenized and freeze-dried, and before the lipids were extracted.

Two human neoplastic tissues were also analyzed. The first was a large right axillary lymph node removed at autopsy and diagnosed to be a lymphosarcoma. The second tissue was a rather slow growing, poorly differentiated sarcoma, removed surgically from an upper arm. These tissues were frozen at –10C and stored before further processing.

In all experiments lipids were extracted according to the procedure of Folch, Lees and Sloane-Stanley (2). The total lipids were measured gravimetrically and then separated into phospholipids and neutral lipids with the use of a silicic acid chloroform slurry (1 g/50 mg lipid). TLC was used to check the completeness of the silicic acid separation.

Isolation of the Suspected Glyceryl Ethers

Because of the availability of the Walker 256 tumor the following procedures were worked out using this tissue.

Column chromatography was performed by the methods of Hirsch and Ahrens (3). The polarity of the unidentified component is closely associated to the polarity of triglycerides with the result that samples containing approximately 70% unidentified lipid and 30% triglycerides were the best attainable with this method.

Preparative TLC plates, coated with 250 μ of Silica Gel G (Applied Science Laboratories, State College, Pa.) and developed in a hexane: diethyl ether (90:10) solvent system, gave the best results for the isolation of the unidentified component. The unknown component was removed from the plates by scraping the area just above the triglycerides. It was essential to scrape the plates and elute the scrapings with chloroform:methanol (1:1) immediately to prevent what appeared to be a breakdown or peroxidation of the component. The eluted samples were pooled and the material again applied to TLC plates for a second isolation which appeared to yield samples in a state of high purity. The component then was stored in chloroform under an atmosphere of nitrogen at –10C until further analysis.

Methods

Infrared analysis was performed with a Perkin-Elmer IR 521 spectrophotometer. The isolated lipids were layered on previously pre-

[1] This work was performed at the Medical Division, Oak Ridge Institute of Nuclear Studies, an operating unit of Oak Ridge Associated Universities, under contract with the U.S. Atomic Energy Commission.

pared 1 cm KBr discs to facilitate their removal for further analysis.

Gas-liquid chromatography (GLC) was carried out on a Wilkens model 204 dual flame ionization instrument equipped with a) a ⅛ in. × 3 ft. column packed with 17% ethylene glycol succinate polyester on 100–140 mesh chromosorb W, and b) a ⅛ in. × 5 ft column packed with 3% QF1 on 100–120 mesh Gas-Chrom Q. The packing material was obtained from Applied Science Laboratories, State College, Pa. The columns were operated between 185C and 205C with a helium flow of 20 to 30 ml/min. Fatty acid standards (NIH) and glyceryl ether standards (obtained through the courtesy of Fred Snyder, Oak Ridge Institute of Nuclear Studies) were used for comparison of retention times. Fatty acid methyl esters were prepared with the use of diazomethane or BF_3-methanol and the glyceryl ether trimethylsilyl (TMS) derivatives were prepared according to the method of Wood et al. (4).

The unidentified lipid was quantitatively analyzed by TLC by means of the densitometric method described by Blank et al. (5).

General Procedures

An infrared analysis was performed on 2 to 5 mg of the lipid isolated from the Walker 256 tumor. The lipid was then washed with chloroform from the surface of the KBr discs and used for saponification.

Saponification. Two methods of saponification were used: a) refluxing 5 ml of a 4% ethanolic KOH solution under nitrogen for 4 hr and b) allowing the unknown lipid component to react in 2 ml of a 0.5N KOH-absolute methanol solution at room temperature under a nitrogen atmosphere for 8 hours; then 1 ml of H_2O was added and the solution allowed to stand for 2 more hours. Two methods of extracting the nonsaponifiable material were used: a) repeated extraction with 3 ml of hexane (this required 10 to 15 extractions to remove all of the nonsaponifiable material) and b) flash evaporation of the basic alcohol-H_2O solutions to dryness in a rotary flash evaporator at room temperature and extraction with chloroform. In both procedures the extracting solvent containing the nonsaponifiables was subsequently dried by addition of Na_2SO_4.

The alkaline solution or basic residue was acidified and the free fatty acids were extracted with hexane. Water was removed from the hexane extracts with Na_2SO_4 and the hexane solution was evaporated to dryness with a stream of nitrogen. Fatty acid methyl esters were prepared and GLC analysis was performed on both polar and nonpolar columns.

Isolation of the Nonsaponifiable Fraction. Thin-layer chromatographic analysis of the nonsaponifiable fraction, with plates coated with Silica Gel G and developed in a hexane: diethyl ether:acetic acid (90:10:1) system, revealed the presence of trace amounts of free sterols, free fatty acids, as well as what appeared to be incomplete saponification of trace amounts of the unknown component. Most of the nonsaponifiable material remained at the origin. TLC with a more polar solvent system of chloroform:methanol (90:10) moved the unknown polar material from the origin to an R_f value of approximately 0.5. A quantitative densitometric analysis was performed in order to evaluate the original composition. The major unsaponifiable fraction was then isolated by preparative TLC with the use of a chloroform:methanl (90:10) system. A chloroform solution of the isolated fraction was applied to the surface of KBr pellets for IR analysis. Finally TMS derivatives were prepared and a GLC analysis was performed.

Isolation and Identification of the Lymphosarcoma Lipid. The techniques used in the isolation and identification of the unidentified lipid in the human lymphosarcoma were essentially those developed and used for the isolation and identification of the lipid present in the Walker 256 tumor. Total lipids were extracted and separated into phospholipids and neutral lipids with the use of a silicic acid chloroform slurry. Thin-layer chromatography was used to check the completeness of the separations. The unidentified lipid class remained with the neutral lipid fraction and was quantitatively analyzed by densitometric procedures. However, unlike the unidentified lipid in the Walker 256 tumor tissue which was isolated prior to saponification the neutral lipid fraction from the lymphosarcoma was saponified directly. The nonsaponifiable material was first extracted with chloroform and then applied to both qualitative and preparative TLC plates and developed in a chloroform:methanol (90:10) system. Free sterols which migrated with the solvent front and the saponified unidentified component which migrated to an R_f of approximately 0.5 were the major materials present. The unidentified component was recovered by scraping the plates and eluting the material with 2:1 chloroform:methanol. Pooled samples were again applied to preparative plates and the material re-isolated in a pure

state as determined by further TLC. After approximately 10 mg were isolated, infrared analysis was performed and TMS derivatives prepared for GLC analysis.

RESULTS

The concentration of the unusual lipid expressed in terms of total lipids ranged from 1 to 3% in five Walker 256 tumors analyzed by the densitometric method while the human lymphosarcoma contained approximately 3.5%. This lipid was not detected by TLC in the lipids extracted from the poorly differentiated human sarcoma.

Thin-Layer Chromatography

The unusual tumor lipid from both the Walker 256 tumor and the lymphosarcoma migrated on TLC plates to the same area as batyl dimyristate (Fig. 1a, lanes 1 to 4). After saponification, the unknown lipid was chemically altered and migrated on TLC plates to the same area as batyl alcohol (Fig. 1b). Quantitative densitometric analysis of the nonsaponifiable material from the Walker 256 tumor showed the major component to be 28% (0.43 mg) of an original sample of 1.55 mg. Considering the original isolated lipid to have a theoretical molecular weight of batyl distearate (875 g moles), the isolated tumor lipid would be approximately 90% pure diacylglyceryl ether. The remaining 10% could be accounted for by the presence of trace amounts of the material that did not saponify and the presence of free sterol-like material (presumably derived from a sterol ester). The latter observation was made when the TLC plates were sprayed with H_2SO_4 and a cherry-red color developed in the region of free sterols.

Infrared Analysis

Fig. 2 shows the results of the infrared analysis of a) the lipid isolated from the Walker 256 tumor, b) the Walker 256 lipid after saponification, c) hexane extract of saponified selachyl diacetate (approximately 70% pure with vitamins A and D and unidentified impurities, Western Chemical, Ltd., Vancouver, Canada) and d) nonsaponifiable fraction isolated using preparative (TLC) from the neutral lipids of a human lymphosarcoma. The significant absorption bands seen in Fig. 3a were 1740 cm^{-1} for ester carbonyl and 1125 cm^{-1} for (–C–O–C–) stretching. After saponification the ester carbonyl absorption disappeared while the absorption in the 1125 cm^{-1} region remained. In addition, there was the appearance of absorption in the 3350 cm^{-1} region depicting (–OH) stretching. These characteristic bands were also seen for the known selachyl alcohol sample (Fig. 2c) and the nonsaponifiable material isolated from the human lymphosarcoma neutral lipids (Fig. 2d).

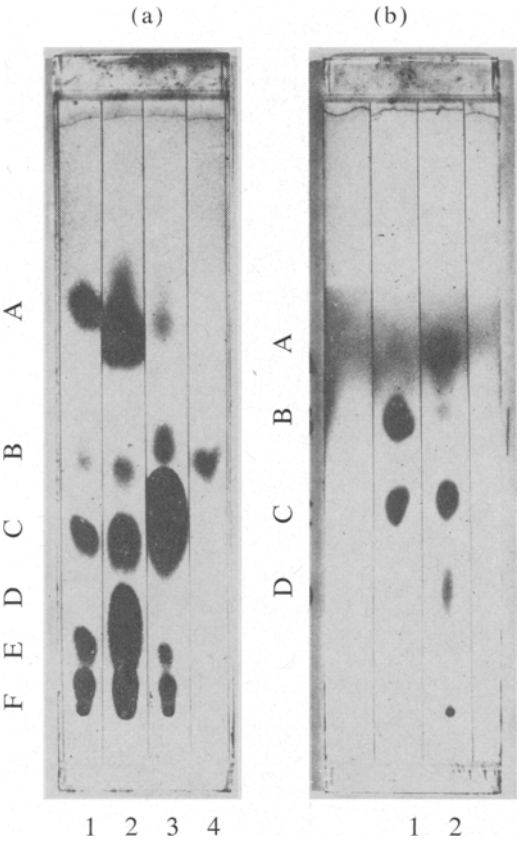


FIG. 1. (a) Thin-layer chromatcgraphic analysis of tumor lipids in a 90:10:1, hexane: diethyl ether:acetic acid solvent system and with plates coated with Silica Gel G. 1) Standard mixture: (a) cholesterol, oleate, (b) batyl dimyristate, (c) triolein, (d) oleic acid, (e) cholesterol, (f) hydrogenated lecithin; 2) Walker 256 neutral lipids; 3) human lymphosarcoma total lipids; and 4) unknown lipid isolated from Walker 256 tumor lipids.

(b) Thin-layer chromatographic analysis of the nonsaponifiable fraction of the unknown lipid on plates coated with Silica Gel G and developed in a 90:10 chloroform:methanol system. 1) Standard mixture of (b) cholesterol and (c) batyl alcohol; 2) nonsaponifiable fraction of unknown tumor lipid, (a) nonpolar lipids and solvent contaminates and (d and below) fatty acid and their potassium salts, respectively.

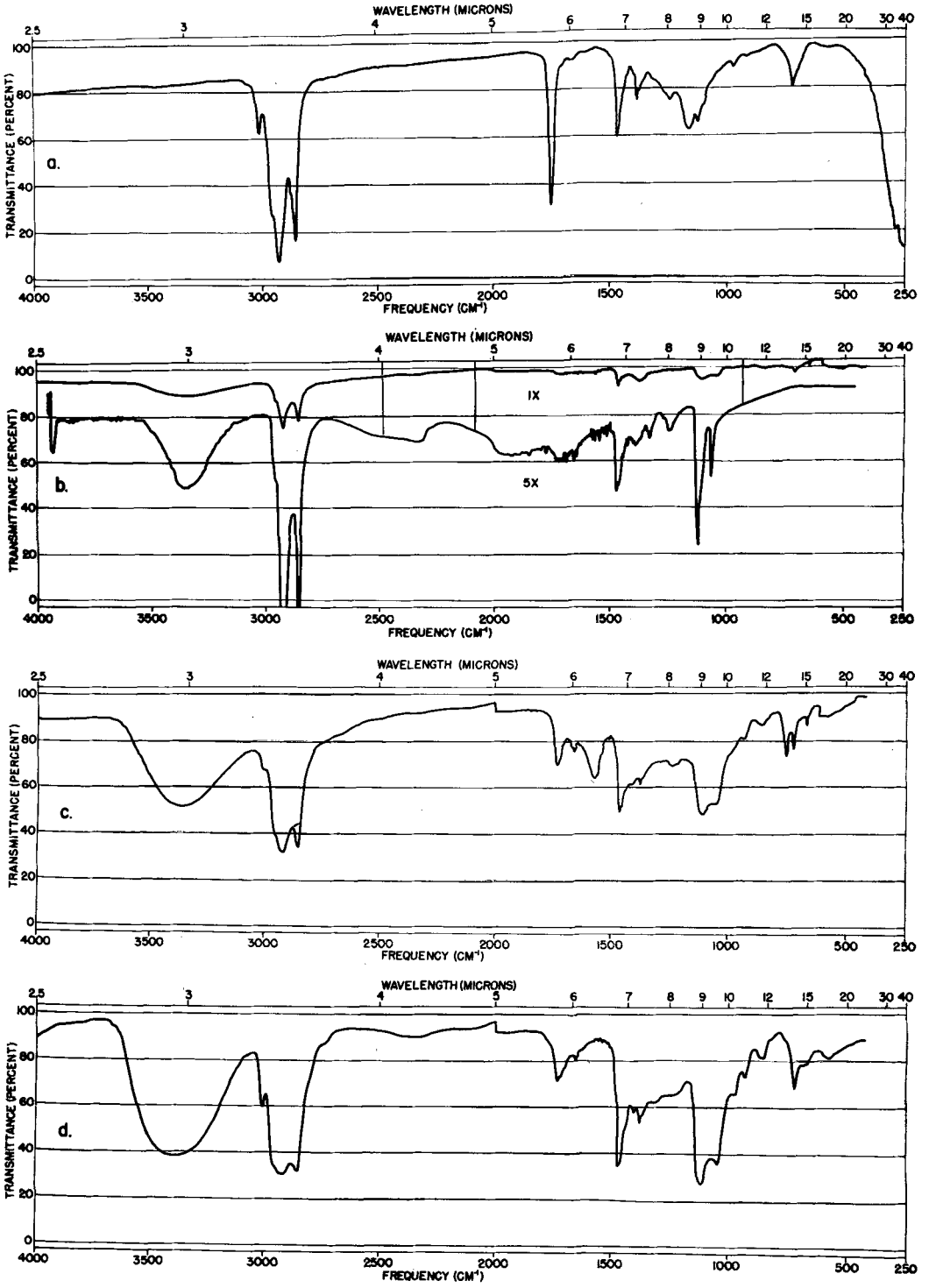


FIG. 2. Infrared analysis of (a) isolated unknown Walker 256 lipid, (b) nonsaponifiable fraction of unknown Walker 256 lipid, (c) selachyl alcohol and (d) nonsaponifiable fraction of the unknown human lymphosarcoma lipid. The instrument used was a Perkin-Elmer IR 521 with the lipid material coated on the surface of KBr discs.

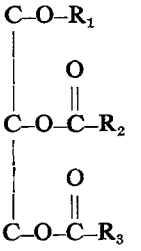
Gas-Liquid Chromatography

Unsuccessful attempts were made to gas-chromatograph the unknown lipid in both the original and saponified form. However, when trimethylsilyl derivatives of the isolated non-saponifiable fraction were made most of the material eluted with the same retention times as TMS derivatives chimyl (C_{16}-glyceryl ether) and batyl (C_{18}-glyceryl ether) alcohols (Fig. 3a). Both these glyceryl ethers are saturated and the unsaturated counterparts were not detected. However, it has recently been shown that the resolution of the TMS derivatives of homologs of such isomers is quite poor (6). Fig. 3b shows the GLC analysis of TMS derivatives of the saponified material isolated from the human lymphosarcoma. This lipid also exhibited the same retention times as chimyl and batyl alcohol-TMS derivatives.

Gas-liquid chromatographic analysis of the fatty acid methyl esters obtained from the isolated Walker 256 tumor lipid was made. The major fatty acids present were palmitic, stearic, oleic and arachidonic acids, with a total of 21 additional fatty acids discernible but not definitely identified. Fatty acids with carbon chain length of C_{16}, C_{18}, C_{20}, C_{22} and possibly C_{24} were shown to be present when the methyl esters were applied to a nonpolar GLC column.

DISCUSSION

This study has tentatively identified the unusual lipid occurring in the Walker 256 tumor of the rat and a human lymphosarcoma to be diacyl derivatives of glyceryl ethers.



R_1 represents a long chain alcohol of 16 to 18 carbons.

R_2, R_3 represent fatty acids of 12 to 22 carbons with varying degrees of unsaturation.

The tentative identification of diacyl derivatives of glyceryl ethers is based on the following: 1) R_f values of the unusual lipid on TLC plates are the same as batyl dimyristate; 2) R_f values of the isolated saponified lipid on TLC plates are the same as batyl alcohol (saponified batyl dimyristate); 3) the molar ratio of fatty acids to glyceryl monoether of the unknown lipid was similar to the theoretical value of that for batyl distearate;

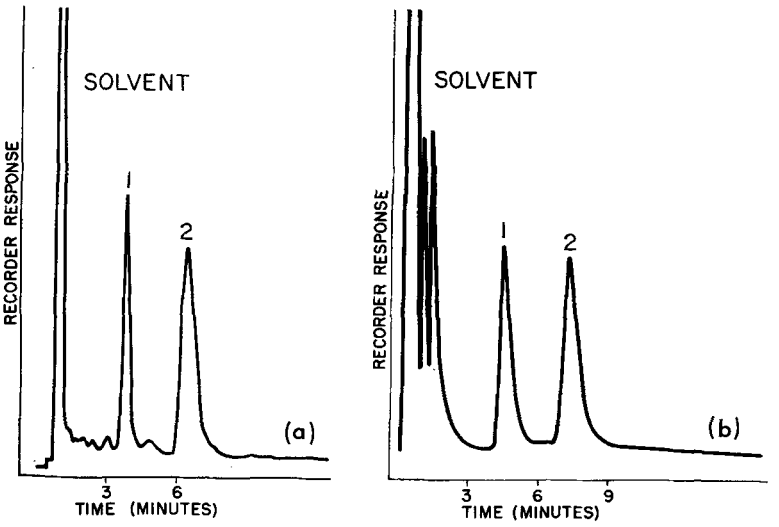


FIG. 3. (a) Gas-liquid chromatographic analysis of trimethylsilyl derivatives of the unknown Walker 256 nonsaponifiable lipid on an EGS column. Retention times were exactly the same as known chimyl (1) and batyl alcohol (2) TMS derivatives.

(b) Gas-liquid chromatographic analysis of trimethylsilyl derivatives of the unknown human lymphosarcoma nonsaponifiable lipid on a QFI column. Retention times were exactly the same as chimyl (1) and batyl alcohol (2) TMS derivatives.

4) infrared spectra of known diacylglyceryl ethers and glyceryl monoethers were the same as those of the unknown lipid and; 5) trimethylsilyl derivatives of the saponified unknown lipid had GLC retention times equal to those of known batyl and chimyl alcohol TMS derivatives.

Since the unusual lipid obtained from the lymphosarcoma was not isolated prior to saponification, as was the case with the lipid from the Walker 256 tissue lipids, the possibility exists that the lipid isolated after saponification could have been due, in part, to the presence of glyceryl monoethers. Analysis of the neutral lipid fraction isolated from the lymphosarcoma tissue (prior to saponification) indicated, however, that either the nonacylated glyceryl monoethers were not present or were not detected by the methods used for visualization of the TLC plates.

It was further noted that the diacylglyceryl ethers which were isolated from the Walker 256 tumor lipids were not as pure as TLC analysis initially indicated. When the isolated lipid was saponified and rechromatographed the solution contained what appeared to be free sterols and some material which was resistant to saponification. Densitometric analysis indicated that components other than diacylglyceryl ethers amounted to about 10% of the original spot. As a result, the concentration of this lipid is slightly less than what the densitometric analysis had initially indicated. However, the corrected concentrations are still much higher than those found for glyceryl ethers in normal mammalian tissue lipids; human marrow (0.2%), spleen (0.05%), red blood cells (0.01%) (7), human liver (0.0%), cow bone marrow (0.01%), and milk (0.01%) (8).

The presence of diacylglyceryl ethers in fairly high concentration (0.9 to 3.2%) in tumor lipids raises several questions in light of what is known about the nonacylated alkoxyethers. Specifically alkoxyglyceryl monoethers have been shown to induce cell proliferation. Linman et al. (9) have shown batyl alcohol to stimulate hemopoiesis as indicated by reticulocytosis, thrombocytosis, leukocytosis, and hyperplasia of all myeloid elements of the bone marrow. Brohult (10) found that the alkoxyglyceryls also enhanced the growth of rats and lactobacillus. In addition, Bodman and Maisin (11) have reported upon the effectiveness of alkoxyglyceryl ethers in the promotion of wound healing. However, any conclusions drawn between these findings and the occurrence of diacyl alkoxyglyceryls in neoplastic tissue would be purely speculative.

This study does indicate the need for more extensive investigation on the role diacylglyceryl ethers play, if any, in neoplastic tissue formation and growth.

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Determination of the Structure of Lecithins via the Formation of Acetylated 1,2-Diglycerides

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ABSTRACT

A detailed procedure for quantitative determinations of molecular species of lecithins is described and applied to several lecithins isolated from natural sources. The method is based on the conversion of lecithin to acetylated 1,2-diglycerides and analysis of these compounds by methodology used for the determination of triglyceride structure.

The preparation of the acetylated 1,2-diglycerides was carried out via hydrolysis with phospholipase C and acetylation of the resultant, 1,2-diglycerides with pyridine-acetic anhydride. Preparation of acetylated 1,2-diglycerides from lecithin by acetolysis with acetic acid-acetic anhydride was shown to be accompanied by intermolecular as well as intramolecular rearrangement of the fatty acids.

The structure of the acetylated 1,2-diglycerides was determined by a combination of argentation-TLC and pancreatic lipase hydrolysis using internal standards for quantification. The method was applied to lecithins isolated from milk serum, egg, soybean, safflower seed and wheat germ lipids.

INTRODUCTION

PARALLELING EARLY METHODOLOGY for the determination of triglyceride structure the first method for determination of classes of lecithins, developed by Privett and Blank (11), was based on cleavage of the unsaturated linkages and analysis of the phosphatidyl choline residues. More recently, Blank, Nutter and Privett (3) described an improved method for determination of lecithin structures based on total fatty acid composition, fatty acids in the β position, and the amount of the disaturated class (determined via mercuric acetate adduct formation). These methods give an analysis of lecithins in terms of positions of saturated and unsaturated fatty acid constituents. A more discriminating analysis by direct application of fractionation techniques to lecithins, particularly argentation chromatography used widely in triglyceride analysis (1,2,17-19), has been generally unsuccessful (5,8).

Renkonen (12,13) was the first to recognize that the solution of the problem of the molecular species analysis of glycerophosphatides, where recovery of the intact compounds was not required, was to convert these compounds to the analogous and less polar acetylated 1,2-diglycerides.

Lecithins may be converted to acetylated diglycerides via acetolysis or via enzymatic hydrolysis with phospholipase C followed by acetylation with acetic anhydride-pyridine of the resulting 1,2-diglycerides. Any migration of acyl groups in the formation of the acetylated 1,2-diglycerides would obviously invalidate the use of these compounds for structural analysis. Renkonen (12), using an acetic acid-acetic anhydride procedure, reported that no significant acyl migration occurred during the acetolysis of glycerophosphatides. However, he indicated in a recent report (14) that some intramolecular rearrangement occurs in the application of this reaction to some glycerophosphatides.

We found (9) that acyl migration of fatty acids occurs in the acetolysis of lecithin by the acetic acid-acetic anhydride procedure. Rearrangement of the fatty acids was demonstrated by differences between the composition of the fatty acids in the β position of the original lecithin determined via phospholipase A hydrolysis and that of the resultant acetylated 1,2-diglycerides determined via pancreatic lipase hydrolysis. A similar comparison made with the acetylated 1,2-diglycerides obtained via phospholipase C hydrolysis and acetylation showed no such differences and it was concluded that not only was there no acyl migration during this procedure but that a valid analysis of the distribution of the fatty acids in the β position of acetylated 1,2-diglycerides could be obtained via pancreatic lipase hydrolysis.

The present studies show that inter- as well as intramolecular rearrangement occurs in the acetolysis of lecithin under the conditions described by Renkonen (12), and emphasize the value of the enzymatic method for the conversion of lecithin to the acetylated 1,2-diglycerides. At present, methods for the de-

termination of structure based on the principle of converting the glycerophosphatides to less polar derivatives have been used mainly for the detection of the major molecular species of these compounds. Noteworthy applications in this respect have been made by van Deenen and his associates (4-6). Particularly pertinent to this study was an application to rat liver lecithin by van Golde, Zwaal and van Deenen (5), in which some 29 different molecular species were detected.

Described here is a detailed procedure for quantitative determination of molecular species of lecithins based on fractionation and analysis of the analogous acetylated 1,2-diglycerides prepared via phospholipase C.

EXPERIMENTAL

Materials

Lecithins were isolated from milk serum (obtained from the Rice Lake Creamery, Rice Lake, Wis., through the courtesy of Donald Gerland), fresh hen eggs, a commercial sample of soybean lecithin (obtained from Nutritional Biochemicals Corporation), crude safflower seed lecithin (supplied by John Kneeland of the Pacific Vegetable Oil Company), and wheat germ oil (obtained from the Viobin Corporation, Monticello, Ill.). The isolation and purification of the lecithins were carried out as previously described (10) by a combination of DEAE cellulose and silicic acid-silicate-aqueous ammonia column chromatography according to the general techniques described by Rouser et al. (15).

Methyl pentadecanoate and other methyl esters used as standards for gas-liquid and thin-layer chromatographic analyses were obtained from The Hormel Institute.

Phospholipase C. The source of this enzyme was the *Cl. Perfringens* Type A toxin (Welchii). A solution of the enzyme with an assay of 400 MLD/ml in the mouse was obtained through the courtesy of Charles Pidacks of Lederle Laboratories, Spring Valley, N. Y.

Pancreatic Lipase. Steapsin obtained from Nutritional Biochemicals Corporation was the source of this enzyme.

Preparation of Acetylated 1,2-Diglycerides

In order to determine if the acyl migration that occurs in the acetolysis of lecithins is entirely intramolecular as reported by Renkonen (12,14), or whether intermolecular shifts of acyl groups also occur simultaneously, a comparison was made of the class composition of the acetylated 1,2-diglycerides prepared from a sample of lecithin by acetolysis and via the phospholipase C hydrolysis procedure (described below). If there is no intermolecular rearrangement, there should be no differences in class compositions of the acetylated 1,2-diglycerides prepared by the two methods; that is, in the amounts of the acetylated derivatives of disaturated (SS), diunsaturated (UU) and unsaturated-saturated (US + SU) classes. Inter- and intramolecular rearrangement cannot be distinguished in the US + SU class by the relative amounts of the two isomers, but the total amount of the two isomers should be the same in the absence of intermolecular rearrangement.

Soybean lecithin containing 58.6% added hydrogenated lecithin (SS) was used in this experiment in order to have a sample that contained an appreciable amount of the disaturated (SS) as well as the diunsaturated class (UU). The acetylated 1,2-diglycerides were isolated by TLC and analyzed for class composition as described previously (3) except that the diunsaturated class was determined by argentation-TLC as described below. The results (Table I) showed considerable differences between the class compositions of the acetylated 1,2-diglycerides prepared via phospholipase C and acetolysis. The rearrangement of the classes during acetolysis does not attain a complete randomization because the concurrent acetylation stabilizes the acyl groups against further migration in the formation of acetylated diglycerides. Comparison of the values for the classes of the acetylated 1,2-diglycerides prepared by acetolysis with those

TABLE I

Class Composition of Acetylated 1,2-Diglycerides of Soybean Lecithin Containing Added Hydrogenated Lecithin [a]

(% by Wt)

Class	Acetylated 1,2-diglycerides prepared *via* phospholipase C-acetylation		Acetylated 1,2-diglycerides prepared *via* acetolysis [b]		Calculated for a random distribution pattern
α β					
S S	56.8		52.1		40.6
U S	0.1	13.6	9.6	24.0	23.1
S U	13.5		14.4		23.1
U U	29.6		24.9		13.2

[a] Class composition determined by the method of Blank, Nutter and Privett, Lipids *1*, 132 (1966), except SS class estimated via argentation-TLC.

[b] Acetylated 1,2-diglycerides prepared by the method of Renkonen, JAOCS *42*, 298 (1965).

prepared via the phospholipase C procedure indicates that the intermolecular rearrangement of the fatty acids had proceeded to the extent of about 25% toward complete randomization. That intramolecular rearrangements also occurred was indicated by the change in the relative amounts of the US and SU classes (Table I).

The method for the conversion of lecithin to acetylated 1,2-diglycerides via phospholipase C hydrolysis is an adaptation of that described by Sribney and Kennedy (16). The sample (10-100 mg) is dissolved in 20 ml of freshly distilled diethyl ether and added to a 50 ml volumetric flask containing 0.5 ml of 0.4 M $CaCl_2$ solution and 1 ml of the phospholipase C enzyme solution (alpha toxin A solution diluted with an equal volume of Tris buffer at pH 7.5). The solution is made oxygen-free by bubbling a slow stream of nitrogen through it, and is stirred vigorously by means of a magnetic stirrer. The course of the reaction is followed by TLC. For this analysis an aliquot of approximately 35 μl is spotted on a 2 in. $\times$ 8 in. chromatoplate coated with Silica Gel G (Merck AG, Darmstadt, Germany) and developed in petroleum ether:ethyl ether (1:1). The spots are visible by charring. The extent of the reaction is determined by the disappearance of lecithin. Generally, the reaction requires 1 to 2 hr for completion.

After the reaction is complete, the contents of the volumetric flask are transferred to a separatory funnel and extracted three times with 50 ml volumes of freshly distilled low-boiling (40-65C) petroleum ether. The petroleum ether extracts are combined, washed several times with distilled water and then dried with anhydrous sodium sulfate.

Acetylation of the 1,2-diglycerides is carried out as follows: the petroleum ether solution containing the diglycerides is reduced in volume to about 2 ml and transferred to a 15 $\times$ 125 mm test tube constructed for sealing; the remainder of the petroleum ether is removed by evaporation in a stream of nitrogen; acetic anhydride, 2.5 ml, and 0.5 ml of pyridine are added to the ampule, which is then sealed; the ampule is heated for 3 hr in a boiling water bath; the solvent is evaporated under reduced pressure at about 50C and the crude reaction mixture is partitioned between 60 ml of chloroform:methanol:water (8:4:3, v/v/v); the acetylated diglycerides separate into the chloroform solution which is separated and dried over anhydrous sodium sulfate for 30 min; the acetylated 1,2-diglycerides are recovered by evaporation of the solvent and purified by thin-layer chromatography with petroleum ether containing 25% diethyl ether on plates covered with Silica Gel G; and the band of acetylated 1,2-diglycerides is scraped from the plate and recovered by extraction with diethyl ether.

Fractionation of Acetylated 1,2-Diglycerides

This step in the procedure is carried out by an adaptation of the general procedure developed for triglycerides in previous work in our laboratory (2). Briefly, the sample is applied in 10 mg amounts to 20 $\times$ 20 cm plates coated with a layer of 0.25 mm in thickness of Silica Gel (Merck AG, Darmstadt, Germany) impregnated with silver nitrate (1) and developed with 0.8% methanol in chloroform.

This solvent generally separates the acetylated 1,2-diglycerides containing 0, 1, 2, 3 and 4 double bonds into separate bands. A 6th band remains on the origin and contains the acetylated diglycerides with acyl groups having higher degrees of unsaturation. The bands are made visible by spraying the plate with a 0.5% ethanol solution of 2,7-dichlorofluorescein. The bands must be marked carefully for quantitative analysis. The front or leading edge of each band will be distinct and must be marked (a sharp pencil is recommended) just above this leading edge. Frequently, a band may tail into the band below it. It is important that none of the lower band be taken with the band above it.

The bands are scraped from the plate into 50-ml beakers, slurried with a 10% aqueous solution of HCl and extracted four times with small volumes of diethyl ether:methanol (3:1). The extracts are filtered through a sintered glass funnel and extracted with distilled water several times to remove the alcohol. The solvent is reduced in volume and the solution of each band diluted to a volume of 5 ml in a glass-stoppered volumetric flask. Should it be necessary, the band at the origin of the plate may be further fractionated by TLC in a like manner with a more polar solvent system.

Analysis of Acetylated 1,2-Diglyceride Fractions

A small aliquot of each solution containing a minimum of about 0.2 mg of sample is transferred to an ampule (15 $\times$ 125 mm test tube constricted for sealing) containing a small but known amount (ideally 25-50% of the sample) of methyl pentadecanoate. The solvent is evaporated in a stream of nitrogen which is

directed into the tube through a hypodermic needle. When the solvent is evaporated and an atmosphere of nitrogen established, about 3 ml of a methanol solution of approximately 5% by weight of HCl is added to the ampule and the ampule is sealed with a torch. It is heated in a boiling water bath for 1 hr or allowed to stand overnight. The methyl esters are then recovered and analyzed by GLC.

In the present work, methyl ester analysis was carried out with an F&M Model 609 hydrogen flame GLC instrument equipped with a 7 ft × ⅛ in column packed with 8% ethylene glycol succinate polyester phase on Gas-Chrom P (Applied Science Laboratory, State College, Pa.). The column is usually operated at 185C, with a carrier gas flow, helium, of 75 ml per min. The fatty acid composition is calculated directly from the proportionalities of the peak areas; the linearity of detector response was established through the analysis of standard mixtures of methyl esters obtained from The Hormel Institute.

From the methyl ester analysis, with reference to the added methyl pentadecanoate, the relative amount of each band is determined. The composition of the molecular species of the acetylated diglycerides of each band is determined from the position of the band on the plate, which gives the number of double bonds per molecule, and the fatty acid composition. As an example of this analysis, two bands, one simple and one complex, obtained in the analysis of the acetylated 1,2-diglycerides of soybean lecithin, are illustrated in detail below. The pertinent data on the bands of the acetylated soybean 1,2-diglycerides are given in Table II.

The amount of material in each band is calculated from the ratio of the sum of the percent peak areas of the components to the peak area of the added methyl pentadecanoate.

TABLE II

Fatty Acid Composition of Acetylated 1,2-Diglycerides of Soybean Lecithin Fractionated by Argentation-TLC (% by Wt)

[a]Fatty acids	Total sample	Bands from the top to the origin on the plate					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
[b]15:0	..	65.6	23.4	36.6	15.8	44.5	85.8
16:0	12.8	12.1	27.1	1.4	3.3	..	..
18:0	3.9	5.5	8.6	..	1.0	..	..
18:1	11.6	16.8	5.1	30.4	1.3	3.6	..
18:2	64.9	..	35.8	31.6	75.4	38.3	6.8
18:3	6.8	..	..	..	3.2	23.6	7.4

[a] Shorthand designation. First number = chain length, number after colon = the number of double bonds.

[b] Added internal standard.

The amount of methyl pentadecanoate added as internal standard to the aliquot (1 ml) of Band No. 1 was 1.3 mg. Therefore, the quantity of Band No. 1 was $34.4/65.6 \times 1.3 \times 5 \left[\frac{34.4 \text{ (peak area of component fatty acids)}}{65.6 \text{ (peak area of standard)}}\right] = 3.5$ mg. The factor 5 is included because the analysis was carried out on a 1 ml aliquot of the sample taken from the 5 ml volumetric flask. The amount of material in other bands is calculated in a similar manner. After calculating the amount of material in each band, the amount of Band No. 1 is found to consist of 4.3% of the total sample.

Band No. 1, which is one of simple composition, consists of the saturated-unsaturated class; that is, a mixture of the α-S, β-M and α-M, β-S (where S = saturated fatty acids and M = monoenoic fatty acids) because, 1) it appeared at a position on the plate corresponding to one double bond per molecule, and 2) the fatty acid composition showed that it contained an equal amount of saturated and monoenoic fatty acids. 17.6% (12.1 + 5.5) and 16.8%, respectively. Methyl acetate that is produced from the acetylated hydroxyl is, of course, eliminated in the work-up of the esters, and the compositions calculated in the above manner represent the actual percent distribution in the original lecithin molecule.

Band No. 3 is contaminated with material tailing into it from the one above it. Its composition can be calculated, providing that it and the other bands are removed from the plate as described above, to insure that no contamination in a band from the one below it on the plate. The position of Band No. 3 on the plate corresponded to compounds having three double bonds, and the fatty acid composition showed that it consisted mainly of a mixture of MD and DM (M = monoenoic acid, oleic, and D = dienoic acid, linoleic). However, since this band also contained a small amount of palmitic acid, it contained a small amount of acetylated diglyceride tailing into it from the band above it (Band No. 2). Examination of the fatty acid composition of Band No. 2 shows that it consisted of DS + SD and MM. The 1.4% palmitic acid in Band No. 3 is due to palmitate in DS + SD. Thus there is also 1.4% linoleic acid (D) in Band No. 3 from Band No. 2. Therefore, 1.4% is subtracted from the value of 31.6% found for linoleic acid to obtain the correct amount of this acid that arises from MD + DM in Band No. 3.

The value that is obtained (31.6 − 1.4 =

30.2) is in good agreement with the value 30.4 found for oleic acid (M) as required for MD + DM. From these results the percentage composition and absolute quantity of MD + DM and SD + DS can be calculated. Had the value for linoleic acid (D) not agreed with that for oleic acid (M), it would then be presumed that this band was also contaminated with MM, the minor component of Band No. 2, and the amount of it, accordingly, would be calculated. It is evident that there was fractionation within Band No. 2 that separated the MM toward the top, and thus the MM did not tail down into Band No. 3, inasmuch as the agreement between the values for the ester analysis in Band No. 3 is within the experimental error of a GLC analysis. The small amount of SD + DS in Band No. 3 is added to that in Band No. 2 to give the total amount of these species in the sample.

All of the bands are calculated in a similar manner. Then another aliquot of each band is taken for determination of the α-β isomer composition of each class as described in the next section.

Determination of Isomeric Species

It is evident from the examples shown above that the molecular species may exist as isomers having the same fatty acid constituents. These isomers can be differentiated via pancreatic lipase hydrolysis. The procedure used for the pancreatic lipase hydrolysis is an adaptation of that described by Mattson and Volpenheim (7). An aliquot consisting of 1–5 mg of each band is transferred to a 10 × 75 mm test tube and the solvent is evaporated in a stream of nitrogen. Then 20–25 mg of tricaprin, 0.9 ml of Tris buffer (adjusted to a pH of 8.0), 20 μl of a 1% solution of bile salts (Ox bile, Nutritional Biochemicals Corporation), 50 μl of a 45% solution of $CaCl_2$ and 8 mg of lipase (steapsin) suspended in 0.1 ml of Tris buffer are added to the test tube and it is placed in a water bath at 40C. A small magnetic stirring bar is added to the test tube and the mixture is stirred vigorously for exactly 15 min. At the end of 15 min, 0.5 ml of 10% aqueous HCl is added to stop the reaction. The mixture is then transferred by washing the tube with a total of approximately 50 ml of ethyl ether into a 125 ml Erlenmeyer flask. Approximately 4 g of anhydrous sodium sulfate is added to this solution and it is allowed to stand for about 30 min. The solution is then decanted, rinsed twice with petroleum ether into a 125 ml suction flask and reduced in volume to about 0.5 ml by evaporation under reduced pressure.

The next step in the procedure is to isolate and determine the fatty acid composition of the 2-monoglycerides. The petroleum ether solution of the products of the lipase hydrolysis is applied to approximately one-half of a 20 × 20 cm chromatoplate coated with Silica Gel G. The monoglyceride band can generally be seen without the aid of an indicator but may be detected by spraying the plate with 2,7-dichlorofluorescein for very small samples. The plate is developed with a solvent consisting of diethyl ether:petroleum ether:acetate acid (30:70:2, v/v/v). The monoglycerides which are near the origin are scraped from the plate directly into an ampule (10 × 125 mm, constricted test tube) in which they are esterified with 3 ml of a 5-6% solution of dry HCl in methanol. The methyl esters are recovered and analyzed by GLC. The isomer composition is then determined on the basis of the fatty acid composition. For example, the fatty acid composition of the β-monoglyceride of Band No. 1, which consisted of 4.3% of SM + MS, was 27.7% saturated (S) and 73.3% monoenoic acids (M). Thus, the percentages of SM and MS in this fraction were (73.3/100 × 4.3) = 3.1% and (27.7/100 × 4.3) = 1.2%, respectively.

RESULTS

Application of the above method to soybean, milk, egg, wheat germ and soybean lecithins are shown in Table III. The molecular species

TABLE III
Structure of Lecithins[a]
(% by Wt)

Type	Milk	Soybean	Egg	Wheat germ	Safflower
$\alpha\beta$					
SS	10.2	..	tr	..	0.5
SM	28.3	3.1	49.3	6.9	4.2
MS	15.8	1.2	0.6	0.7	0.3
MM	17.1	1.8	3.2	2.5	1.4
SD	8.2	24.0	31.6	28.4	34.7
DS	2.0	1.5	0.1	0.8	1.1
MD	3.9	8.9	3.5	11.8	8.0
DM	3.7	5.9	0.8	4.0	5.0
S-TRI	2.1	3.5	..	1.7	..
TRI-S	0.7	0.4	..	..	..
DD	0.5	39.7	..	36.3	44.8
D-TRI	..	1.8	..	2.0	..
TRI-D	..	6.9	..	3.6	..
M-TRI	1.5	1.3	..	0.4	..
TRI-M	0.5	tr	..	0.9	..
S-TET	1.6	..	9.9	..	..
TET-S	0.3	..	0.2	..	..
Others	3.6	..	0.8	..	..

[a] S = saturated; M = monoene; D = diene; TRI = triene; TET = tetraene.

were not expressed in terms of specific fatty acids because, although the fatty acid composition was determined, the separations were carried out on the basis of the degree of unsaturation. Thus, although the major monoene, diene and tetraene are oleic, linoleic and arachidonic acids, respectively, minor amounts of other isomers are present and it would be incorrect to exclude them. The present procedure is, in fact, so designed that only a minimum amount of sample is used. Aliquots are left for further analysis via gas-liquid and liquid-liquid partition chromatography. The application of these techniques to acetylated diglycerides for incorporation in a more extensive scheme of analysis for lecithin structures is currently under investigation.

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Metabolism of Unsaturated Fatty Acids in the Intestine

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ABSTRACT

Rats fed a fat-free diet from weaning were continued on that diet alone or supplemented with methyl linoleate, methyl linoleate plus a mixture of antibiotics, or methyl arachidonate.

Dietary linoleate and arachidonate reduced the concentration of octadecenoic acid and increased that of stearic acid in the mucosa and luminal lipids. This effect was prevented in the mucosa but not in the intestinal contents by antibiotic supplementation of the linoleate diet.

Evidence for the conversion of linoleic into eicosatetraenoic acid was found in both mucosa and luminal lipids. The conversion was impaired by the addition of antibiotics to the diet.

Linoleate feeding combined with antibiotic addition provided evidence for the intestinal hydrogenation of dietary linoleic into either octadecenoic or stearic acids by separate routes, the latter being impaired by antibiotic ingestion.

The ingestion of methyl linoleate or arachidonate modified only slightly the fecal fatty acid pattern of rats previously on a fat-free diet.

INTRODUCTION

THE HYDROGENATION of linoleic and linolenic acids by ruminants was first demonstrated by Reiser (1) and later confirmed by others. Hartman et al. (2) have postulated that a similar phenomenon may take place in the intestine of nonruminants. In general, hydrogenation has been attributed to the microorganisms present in the digestive tract of both ruminant and nonruminant. Blomstrand et al. (3), however, were able to demonstrate the hydrogenation of elaidic acid in the liver of germ-free rats.

The elongation and desaturation of linoleic acid to arachidonic acid in the intestine of the rat has been demonstrated recently (4).

The objectives of this investigation were to test whether hydrogenation actually occurs in the intestine of a monogastric mammal and to ascertain the cause and site of the elongation-desaturation process.

PROCEDURES

Weanling male albino rats of the Institute's strain were fed a diet containing 77% sucrose, 19% acetone-extracted casein, salts and vitamins (5). The diet contained only 0.2% fat and will hereafter be designated as fat-free diet.

When the rats reached a body weight of 140–170 g they were distributed into groups and fed as indicated in Table I. Linoleic and arachidonic acid methyl esters were given by mouth as drops, twice a day at 9 AM and 4 PM. The dose was 120 mg/day/rat. Methyl linoleate (83% pure by gas chromatography, main contaminant, oleic acid 14%) was prepared from sunflower oil by the method of Parker et al (6). Methyl arachidonate was prepared from hog liver (5). The final product was 73% pure, and contained 9% of a contaminant of retention time (in a polar gas chromatographic column) slightly longer than that of arachidonic acid. Nine other contaminants were present, none at a level higher than 4%.

The antibiotic mixture was made up of tetracycline, neomycin, and nystatin (4:4:1, w/w) and was administered by mouth at a rate of 45 mg/day/rat[2] immediately after the esters were fed.

In order to have the dietary supplements mixed with the food in the digestive tract, the rats were not allowed to eat for 4 hr before 9 AM and 4 PM. They were then given the diet for half an hour, at which time the esters were administered.

For 10 days prior to the supplementation period feces were collected from all animals, freed from spilled food, weighed and stored at 4C. The fat was then extracted by the procedure of Van de Kamer et al. (8). Beginning one day after the supplementation of the diets had begun, the feces from each group were collected and treated as indicated above. An aliquot of the feces from the group fed antibiotics was also examined for microorganisms. Very few were detected after 24 hr of

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[2] Assuming a food consumption of 15 g/day/rat, this corresponds to a proportion of antibiotics of about 3,000 ppm. This is slightly higher than a therapeutic dose, which is up to about 2,000 ppm according to Goldberg (7).

TABLE I
Fecal and Luminal Lipids of Rats Fed Polyunsaturated Esters

Dietary supplement	No. of animals	Feces			Intestinal contents
		Feces excreted	Lipid in feces	Lipid excreted	Lipid content[b]
		mg/rat/day	%	mg/rat/day	mg/rat
None[a]	5	550	3.7	21	2
Me-linoleate (8 days)	6	377	3.5	13	4
Me-linoleate (15 days)	7	347	3.4	13	4
Me-arachidonate (8 days)	5	401	3.9	16	5
Me-arachidonate (15 days)	4	444	c	c	c
Me-linoleate + antibiotics (8 days)	4	520	c	c	c

[a]Basic diet containing sucrose 77%, acetone-extracted caseine 19%, salts and vitamins.

[b]After 15-20 hrs. fasting.

[c]Not determined.

incubation (9). However, this could be due to the presence of unabsorbed antibiotics in the feces since after a 48-hr incubation a diluted sample did give a positive bacterial test.

At the end of the feeding period the rats were fasted for 15 to 20 hr, and then killed with ether. The lipids in the intestinal lumen (from the duodenum to the ceceum, inclusive) were extracted by alternate flushings with petroleum ether and physiological saline (10, 11). The washings were acidified and the ether phase decanted and evaporated to dryness. After these washings the small intestine, from the duodenum to the ileum, inclusive, was cut longitudinally and the mucosa scraped off with a scalpel.

The fat in the mucosa was extracted with 2:1 (v/v) chloroform:methanol mixture (12). The extracted lipids were then refluxed in a 2% solution of HCl in absolute methanol. The resulting fatty acid methyl esters were analyzed by gas-liquid chromatography. A Pye argon gas chromatograph equipped with a beta-ionization detector was used. Diethylene glycol adipate (10%, w/w) and Apiezon grease (10%, w/w) coated on 80–100 mesh washed celite were used as liquid phases in columns 4 ft by ¼ in. Temperatures of 180C and 205C were used in each column with a gas flow rate of 60 ml/min. Identification of the peaks was made by comparison of their relative retention times with those of known standards when available. Further evidence of identity was obtained by hydrogenation, using PtO_2 as catalyst (13) followed by gas chromatography in both types of columns. Gas chromatography quantitation data were calculated as area percent. Under analogous conditions the analysis of a sunflower oil standard[3] showed a relative error of less than 8% for major components (10% of total mixture) and less than 33% for minor components (10% of total mixture).

RESULTS AND DISCUSSION

Dietary Balance

Since all animals were fed the same basic diet plus forcefed supplements, it can be safely assumed that they ate similar amounts of food.

The data in Table I show that there were no significant differences among groups with respect to lipid excretion or to the amount of lipid found in the intestinal lumen. Krondl et al. (14) have reported that feeding 30 mg/day chlortetracycline or neomycin to rats does not alter significantly the rate of disappearance of fat from the intestine. In the present experiment, no diarrhea was observed in the animals fed antibiotics. It seems reasonable to assume then, that at the level of antibiotics fed, very little alteration of the lipid balance, if any, was produced.

The degree of absorption of the methyl esters given can be estimated by comparing the amount of lipid excreted per day, which was 13 and 16 mg for the groups fed linoleate and arachidonate, respectively, with the amount of lipid ingested daily: 120 mg. The high absorbability of the esters given is in accordance with data in the literature (15,16).

[3] Division de Matières Graises, Union Internationale de Chimie Pure et Appliqué, 1963 program.

TABLE II

Fatty Acid Composition of the Intestinal Mucosa Lipid of Rats Fed Polyunsaturated Esters

		Dietary supplements[a]			
		Me-linoleate		Me-linoleate + antibiotic	Me-arachidonate
Fatty acid[b]	Fat-free diet	8 days	15 days	8 days	8 days
			percent		
14:0	2.6	0.7	0.4	0.4	0.6
15:0 br	tr	0.5	0.1	tr	0.1
15:0	—	0.7	0.3	0.2	—
15-CP ?	0.9	—	—	—	0.3
16:0 iso-br	0.5	0.4	0.1	0.2	0.2
16:0	*21.3*	*18.1*	*18.7*	*20.6*	*19.8*
16:1	*8.2*	*4.7*	*3.8*	*5.8*	*5.3*
17:0 anteiso-br	tr	—	tr	—	0.2
17:0	—	0.4	0.6	—	0.4
17-CP ?	1.3	—	—	—	—
18:0 iso-br	0.5	0.7	0.5	0.6	0.5
18:0	*13.8*	*18.3*	*18.9*	*14.3*	*15.9*
18:1	*32.4*	*22.5*	*18.4*	*30.4*	*23.2*
18:2	*1.8*	*12.5*	*13.7*	*12.3*	*0.9*
19-CP ?	1.6	—	—	—	—
20:0 iso-br	tr	0.8	—	0.6	—
20:0	tr	1.1	0.8	0.4	0.3
20-CP ?	0.8	—	—	—	—
20:1	2.0	2.7	1.9	2.3	0.9
20:3	tr	2.3	1.4	1.1	0.5
20:3[c]	*8.8*	*1.3*	*0.9*	*4.4*	*0.2*
20:4	*3.3*	*9.1*	*14.1*	—	*27.1*
22:0 iso-br	0.2	—	—	—	—
22:0	tr	0.9	0.9	0.4	2.1
22:1	—	—	1.3	0.4	0.3
23:0 anteiso-br	—	—	0.1	—	—
23:0	—	—	0.3	—	—
22:3	—	—	—	0.3	—
22:5	—	—	—	0.5	—
22:6	—	—	—	0.8	—
24:0 iso-br	—	—	0.6	—	—
24:0	—	1.9	1.2	—	—
24:1	—	—	0.5	—	—

[a]Polyunsaturated methyl esters fed at a dose of 120 mg/day/rat. Antibiotics fed at a dose of 45 mg/day/rat. Major fatty acids are in italics.

[b]br = branched CP = cyclopropane acid

[c]Eicosatrienoic acid with similar retention time to that increased in rat tissue lipids by low-fat diets.

Mucosa Fatty Acids

Since, as indicated above, lipid intake, absorption and excretion are similar in all groups, the effects of supplementation of the diet with methyl esters of individual fatty acids can be followed in the mucosal, luminal, and fecal lipids by examining the fatty acid composition of these lipids.

Table II presents the fatty acid composition of the intestinal mucosas of the various groups. Compared to the fat-free diet, linoleate feeding caused an increase of linoleic acid (from about 2 to 13% in 8 days) and of eicosatetraenoic acid from 3 to 9% in 8 days. Arachidonate feeding increased the concentration of eicosatetraenoic acid from 3 to 27% in 8 days. Arachidonic acid was apparently unchanged, whereas part of the linoleic acid was elongated. However, this elongation did not take place when linoleate and antibiotics were given.

Hexadecenoic, octadecenoic and eicosatrienoic acids are known to abound in the lipids of animals fed no essential fatty acids and to decrease upon the addition of essential fatty acids to the diet (17). In the present experiment, almost 50% of the mucosa fatty acids in the group fed no fat consisted of hexadecenoic (8%), octadecenoic (32%), and eicosatrienoic (9%) acids. The levels of these acids decreased more than could be accounted for by a simple dilution effect when linoleate or arachidonate was fed, while the level of stearic acid increased. Upon feeding linoleate plus antibiotics, neither the decrease in octadecenoic acid nor the increase in stearic acid were noted. This suggested first, that the decrease and the increase were related to each other, and secondly, that linoleate ingestion promoted the hydrogenation of octadecenoic to stearic, or inhibited the dehydrogenation of

TABLE III

Fatty Acid Composition of the Intestinal Content Lipid of Rats Fed Polyunsaturated Esters

		Dietary supplements[a]				
		Me-linoleate		Me-linoleate + antibiotic	Me-arachidonate	
Fatty acid[b]	Fat-free diet	8 days	15 days	8 days	8 days	15 days
		percent				
14:0	2.3	2.8	1.3	1.1	3.5	1.6
14:1	—	—	—	—	—	2.0
15:0 br	0.6	tr	1.4	1.0	tr	tr
15:0	0.5	2.3	0.7	0.3	1.4	0.7
16:0 iso-br	0.3	0.4	0.4	0.4	0.4	tr
16:0	*23.9*	*22.9*	*23.2*	*22.0*	*24.4*	*24.7*
16:1	*13.4*	*6.7*	*6.1*	*8.2*	*10.4*	*8.8*
17:0 br	tr	tr	tr	tr	tr	tr
17:0	0.3	2.6	0.4	0.4	3.9	tr
18:0 iso-br	0.4	tr	0.4	0.5	tr	tr
18:0	*4.1*	*9.2*	*7.8*	*8.9*	*6.6*	*5.2*
18:1	*46.9*	*23.6*	*19.3*	*27.5*	*34.9*	*25.8*
18:2	—	*7.3*	*14.6*	*7.5*	—	—
19:0	1.0	tr	tr	0.1	tr	tr
19-CP?	0.4	—	—	—	—	—
20:0 iso-br	0.3	1.2	1.0	1.4	1.2	1.7
20:0 anteiso-br	0.2	—	—	—	—	—
20:0	0.3	2.5	0.5	0.7	1.8	—
20:1	1.7	4.3	2.8	4.0	2.3	7.6
21:0 anteiso-br	—	2.3	1.0	1.5	—	1.5
21:0	2.1	—	—	0.3	—	—
20:3	—	1.6	0.5	—	tr	—
20:3[c]	—	1.0	0.8	—	tr	3.6
20:4	*1.0*	*6.8*	*13.0*	—	*9.2*	*14.1*
22:0 iso-br	0.3	—	0.4	3.3	tr	—
22:0	tr	tr	0.5	2.0	—	—
?	—	—	2.8	—	—	—
24:0 iso-br	—	—	—	1.7	—	—
24:0	—	—	—	4.7	—	—
?	—	—	—	1.9	—	—

[a]Polyunsaturated methyl esters fed at a dose of 120 mg/day/rat. Antibiotics fed at a dose of 45 mg/day/rat. Major fatty acids are in italics.

[b]br = branched CP = cyclopropane acid.

[c]Eicosatrienoic acid with similar retention time to that increased in rat tissue lipid by low-fat diet.

stearic into octadecenoic, or both. The conversion was sensitive to antibiotics.

Lumen Fatty Acids

Table III shows the fatty acid composition of the lumen lipids of the group fed no fat and the changes induced by feeding methyl linoleate and methyl arachidonate. These changes are quite similar to those detected in the mucosa, except that the decrease in octadecenoic acid and the increase in stearic acid induced by dietary linoleate as compared to the fat-free diet took place even in the presence of antibiotics. These observations suggest the existence of two mechanisms for the simultaneous decrease in octadecenoic acid and increase in stearic acid which result from linoleate administration. One is shown by the acids in the mucosa, and is sensitive to antibiotics. The other, shown by the luminal lipids, is not affected by antibiotic feeding.

As in the mucosa, the eicosatetraenoic acid of the lumen lipids increased noticeably with respect to the group fed no fat after linoleate feeding, but not after linoleate plus antibiotic administration. Thus, the results from both the mucosa and the lumen provide evidence for the conversion of linoleic to eicosatetraenoic acid. Whether this elongation originates in the lumen, the mucosa, or elsewhere, cannot be deduced from the present data.

Since rat enteric bacteria and fungi (18) seem to be unable to produce polyunsaturated fatty chains of 20 or more carbon atoms (19), mucosal cell enzymes are most probably responsible for the elongation.

Supplementation of the ration with a mixture of tetracycline, neomycin and nystatin prevented the elongation from taking place. Which of the three antibiotics is active in this respect cannot be determined from the present data. However, nystatin has been shown to act on membrane sterols and phospholipids (20,12), and it seems quite possible that it

TABLE IV
Fatty Acid Composition of the Fecal Lipid of Rats Fed Polyunsaturated Esters

Fatty acid[b]	Fat-free diet	Dietary supplements[a]				
		Me-linoleate		Me-linoleate + antibiotic	Me-arachidonate	
		8 days	15 days	8 days	8 days	15 days
		percent				
14:0	1.1	0.8	0.6	0.7	0.4	0.9
15:0 br[c]	2.9	1.1	1.1	1.1	1.0	1.4
15:0	5.9	4.3	3.9	0.8	3.6	3.9
16:0 iso-br	1.1	0.9	0.6	0.9	1.0	1.3
16:0	*18.8*	*13.4*	*10.4*	*15.4*	*16.5*	*17.5*
16:1	1.4	1.2	1.0	2.5	1.8	1.8
17:0 br[c]	tr	tr	tr	—	tr	tr
17:0	1.3	1.2	1.0	0.3	1.0	1.3
18:0 iso-br	1.3	1.1	0.7	0.9	1.1	1.4
18:0	*12.7*	*13.2*	*17.0*	*9.8*	*12.1*	*14.2*
18:1	*9.4*	*19.3*	*25.7*	*17.7*	*8.0*	*9.6*
19:0 br[c]	tr	tr	tr	—	tr	tr
18:2	—	*9.0*	*11.6*	*13.6*	*1.0*	*0.7*
19:0	—	—	—	—	tr	tr
20:0 iso-br	*5.3*	*2.8*	*1.8*	*3.5*	*3.5*	*3.0*
20:0	*3.2*	*3.0*	*3.1*	*0.9*	*3.6*	*2.3*
20:1	*8.1*	*5.5*	*4.5*	*6.3*	*9.0*	*7.4*
21:0 iso-br	1.4	tr	tr	1.4	—	—
21:0 anteiso-br	2.8	1.7	1.2	1.4	3.0	1.5
21:0	tr	tr	tr	1.6	—	1.1
20:3	—	—	—	—	1.7	1.7
20:3[d]	—	—	—	0.3	1.8	—
20:4	—	*tr*	*0.6*	*tr*	} *6.9*	2.6
22:0 iso-br	3.3	2.4	0.7	3.4	}	*4.9*
22:0	2.7	3.3	2.4	2.0	4.2	4.3
22:1	3.6	3.0	2.7	1.3	2.1	2.2
23:0 iso-br	tr	tr	tr	—	—	—
23:0 anteiso-br	tr	tr	tr	tr	0.5	tr
23:0	tr	0.9	0.6	tr	0.6	tr
24:0 iso-br	*3.4*	*2.8*	*1.7*	*3.9*	*3.3*	*3.5*
24:0	*4.6*	*6.1*	*5.0*	*7.0*	*8.8*	*7.4*
24:1	*5.7*	*3.0*	*2.1*	*4.8*	*3.5*	*3.8*
25:0 iso-br	tr	tr	tr	tr	tr	tr
25:0 anteiso-br	tr	tr	tr	tr	tr	tr

[a]Polyunsaturated methyl esters fed at a dose of 120 mg/day/rat. Antibiotics fed at a dose of 45 mg/day/rat. Major fatty acids are in italics.

[b]br = branched.

[c]Two isomers present.

[d]Eicosatrienoic acid with similar retention time to that increased in rat tissue lipids by low-fat diet.

could also have some effect on polyunsaturated acids associated with those lipids.

Fecal Fatty Acids

The fatty acid composition of the fecal lipids is shown in Table IV. When comparing these data with those in the previous tables it should be borne in mind that the mucosa and intestinal content figures reflect compositions at a given time, whereas the present ones correspond to samples gathered during periods of several days.

James and co-workers (22-24) have characterized many of the fatty acids of up to about 18 carbon atoms present in human stools. In the present work fatty acids of up to C_{25} were demonstrated in rat feces. These acids appeared in the form of a series of repeated sequence from C_{15} to C_{25} (Table IV). The repeated sequence was as follows: a) odd iso branched chain acid; b) odd anteiso branched chain; c) odd straight chain; d) even iso branched chain; e) even straight chain; f) even straight chain monounsaturated acids. The detection of C_{26} acids in some samples suggest that the series could be even more extensive. Minor proportions of hydroxystearic acid were observed in practically all the fecal samples. The similarity of this fatty acid population to that of the bacterial lipids (19) does not need to be stressed.

Variations in the diet were less well reflected in the fecal lipids than in those from the other media analyzed. However, some changes were apparent. In spite of the high degree of absorbability of methyl linoleate (15) its feeding caused an increase in octadecadienoic acid of the feces from none in the fat-free group up

to about 12% in 15 days (Table IV). Similarly, eicosatetraenoic acid increased when fed, but to a lower level of about 5% in 15 days.

The low concentration (9%) of octadecenoic acid in the feces of rats fed no fat is striking, especially when compared to the levels in the mucosa (32%) and in the intestinal contents (47%). James et al. (23) demonstrated the presence of several isomeric octadecenoates in human stools, and it might well be that the isomer which predominates in the feces is different from that which predominates in other tissues (25). It can be seen in Table IV that feeding of linoleate substantially increased the level of fecal octadecenoic acid from 9% in the group fed no fat to 26% after 15 days of supplementation. Stearic acid also increased, but at a slower rate (from 13 to 17% in 15 days). The addition of antibiotics to the linoleate diet caused a similar increase in octadecenoic acid, with respect to the groups fed no fat, as methyl linoleate alone. However, a higher increase in unchanged linoleic acid, and a decrease in stearic acid were provoked.

The hydrogenation of polyunsaturated acids has been repeatedly demonstrated in ruminants, but only postulated in nonruminants (2). In the present experiment, the marked increase of linoleic acid and less unsaturated counterparts after linoleate ingestion indicates that part of the dietary linoleate was hydrogenated while passing through the intestine. Considered together with the results after antibiotic supplementation they also point to the existence of two mechanisms for this hydrogenation. One route results in the conversion of octadecadienoic to octadecenoic acid and is insensitive to antibiotics; the other transforms octadecadienoic into stearic acid and is inhibited by antibiotics. The latter is then most probably due to microorganism action. In vitro work with rumen bacteria led Polan et al. (26) to postulate the existence of two different systems for the hydrogenation of linoleic acid. The results reported above agree with their data in indicating that stearic acid is formed at a slower rate than octadecenoic acid. Results presented by Hoet et al. (27) also show that the administration of other antibiotics induces changes in the levels of stearic acid and its monoene in the feces.

As compared to the group fed no fat, the concentration of eicosatetraenoic acid in the feces increased after both linoleate and arachidonate administration but not to the levels observed in the mucosa and the intestinal contents. It seems possible that some of the dietary arachidonic acid might have been converted into tetracosanoic acid, in view of the raised levels of this acid in the feces of the animals which received arachidonate. The data in Table IV provide no indications of hydrogenation of dietary arachidonate without elongation.

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Chemical and Physical Properties of Isomeric Glyceryl Monoethers[1]

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ABSTRACT

Long-chain saturated and mono- and diunsaturated 1- and 2-glyceryl monoethers were synthesized by reacting 1,2-isopropylidene and 1,3-benzylidene glycerol potassium salts with alkyl halides in the preparation of the saturated monoethers, and with alkenyl-*p*-toluenesulfonates in the preparation of the unsaturated monoethers, followed by hydrolysis of the blocking groups with boric acid. The progress of the reaction was monitored by gas-liquid chromatography (GLC) of the reaction mixture. The 2-glyceryl ethers, with two exceptions, had not been prepared previously. Normal propyl and 3-pentyl octadecyl ethers also were synthesized to aid in the interpretation of infrared (IR) and nuclear magnetic resonance (NMR) spectra. All the ethers prepared were purified by preparative thin-layer chromatography (TLC) and crystallization. Their purity was found to be greater than 95%, as determined by TLC and GLC, supported by NMR and IR spectra. The isomeric 1- and 2-glyceryl ethers were separated on Silica Gel G adsorbent layers impregnated with either sodium arsenite or boric acid and their TLC behavior interpreted, based on the polarity of the complexes formed. Melting point determinations indicated more than one polymorphic form. Comparison of IR and NMR spectra of the saturated and unsaturated isomeric glyceryl ethers, and various derivatives, demonstrated the applicability of these spectroscopy methods for characterization and structural determination, in addition to distinguishing between the two isomeric forms.

INTRODUCTION

GLYCERYL ETHERS CAN EXIST in two isomeric forms: The hydrocarbon chain can be linked to the one-position of the glycerol molecule (1-isomer) or the two-position (2-isomer). The former is unsymmetrical and can exist as either of two enantiomeric forms, but the latter is symmetrical and can exist in only one form. Heilbron and co-workers, in a series of investigations (1-4), established that the naturally occurring glyceryl ethers found in the oils of elasmobranch fish are the 1-isomer. Baer et al. (5-7) demonstrated by comparison with synthetic enantiomers of the 1-glyceryl ethers that the naturally occurring ethers found in elasmobranch fish oils possessed the D configuration.

Since these early investigations, glyceryl ethers, which were once thought to be exclusively associated with the oils of various marine animals, have been found in many mammalian tissues and fluids, vegetable oils, and domestic hen eggs (8-21) as diesters and phosphatides. Owing to the low concentrations in which the glyceryl ether usually occurs in mammalian tissues and fluids, many investigators assumed without adequate proof that only the 1-isomer was present. The inability to distinguish between the two isomers with microgram quantities reflects the inadequacy of the present analytical methods. Until recently (22), a method did not exist for the simultaneous determination of the 1- and 2-glyceryl ethers in a mixture. The recorded physical and chemical properties of the 1-glyceryl ethers are scant, and the purity of the material used to make the determinations in some cases is questionable. Baumann and Mangold (33) have prepared a number of 1-isomers in a high state of purity and have determined their melting points and critical solution temperatures. The physical and chemical properties of the 2-glyceryl ethers, like the 1-isomer, are even more inadequately known, since only two of these isomers have been prepared and purified.

This lack of basic knowledge of the isomeric glyceryl ethers led us to make a detailed comparison of the physical and chemical properties of several purified synthetic saturated and mono- and diunsaturated 1- and 2-glyceryl ethers and some of their derivatives. The results obtained by comparison of infrared (IR) and nuclear magnetic resonance (NMR) spec-

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tra, melting points, and gas-liquid chromatography (GLC) retention times for both isomers of the various glyceryl ethers are reported here.

ANALYTICAL METHODS

Analytical Gas Chromatography

The gas-chromatographic system and operating parameters used in this investigation have previously been described (22). Column specifications and operating conditions used for the analysis of the isomeric glyceryl ethers, starting materials, and synthetic intermediates are shown in Table I. Columns were packed, tested, and conditioned thermally and with trifluoroacetic anhydride before use (22). The information obtained from the GLC analyses of the compounds shown in Table I, other than the isomeric glyceryl ethers, which have been shown to give quantitative results (22), was used in a qualitative or semiquantitative manner only. Quantitative results were calculated from peak area measurements determined by triangulation.

Preparative Gas Chromatography

An Aerograph Model 90-P3 gas chromatograph (Wilkens Instruments and Research, Inc., Walnut Creek, Calif.), equipped with a thermal conductivity detector, was used for this purpose. Column specifications and operating conditions used for the fractionation and collection of the various compounds are shown in Table I (bottom). A flow rate of approximately 200 ml/min of He carrier gas was used. A fraction collector previously described by Wood and Reiser (23) was used for the collection of desired components.

Nomenclature

A shorthand system of nomenclature used to identify the isomeric glyceryl ethers has previously been described in detail (22). It is essentially a numerical system, the first number

TABLE I

Column Specifications and Operating Conditions Used for the Analysis of Glyceryl Ethers, Starting Materials and Synthetic Intermediates

Compound analyzed	% Liquid phase	Support	Column dimensions[a]	Column temp (°C)
		Analytical		
Glyceryl ether TFA deriv.	10.5 Ethylene glycol succinate methyl silicone polymer (EGSS-X)[b]	Gas-Chrom P 100-120 mesh	5 ft × ⅛ in. SS and Al	170
Glyceryl ether TFA deriv.	5 Methyl silicone polymer (SE-30)	Chromosorb W 60-80 mesh[c]	5 ft × ⅛ in. SS[d]	230
Glyceryl ether isopropylidene deriv.	16 Ethylene glycol succinate polyester (EGS)[b]	Chromosorb W 100-140 mesh	3 ft × ⅛ in. Al	220
Alkyl iodides and bromides	16 EGS	"	10 ft × ⅛ in. Cu and Al	185
n-propyl Octadecyl ether	"	"	3 ft × ⅛ in. Al	190
3-pentyl Octadecyl ether	"	"	"	200
Oleyl and linoleyl alcohol TMS ether deriv.	"	"	"	150
Oleyl- and linoleyl-p-toluene sulfonates	"	"	"	210
		Preparative		
18:1-1 TMS ether deriv.	25 SE-30[e]	Gas-Chrom Rz[b] 50-60 mesh	7 ft × ⅜ in. Al	285
n-propyl Octadecyl ether	"	"	10 ft × ⅜ in. Al	200
3-pentyl Octadecyl ether	"	"	"	265

[a] Columns were stainless steel (SS), Aluminum (Al) and Copper (Cu).

[b] Obtained from Applied Science Laboratories, State College, Pa.

[c] Acid washed support.

[d] Pretested packed columns obtained from Wilkens Instrument & Research, Inc., Walnut Creek, Calif.

[e] Liquid phase coated on support by flash evaporation technique.

represents the number of carbon atoms in the hydrocarbon chain, the second number represents the number of double bonds in the chain and the third denotes to which carbon atom of the glycerol molecule the hydrocarbon chain is bonded. The sometimes used α and β system of denoting isomers is synonymous with the 1 and 2 systems used here.

Infrared Spectroscopy

Infrared spectra were obtained with a Perkin-Elmer (Perkin-Elmer Instrument Div., Norwalk, Conn.) Model 521 Grating Infrared Spectrophotometer. Spectra of solvent-free liquids were obtained by sandwiching between two optically-ground potassium bromide (KBr) crystals. Spectra of the solid compounds, and sometimes of liquids, were obtained with 300 mg KBr discs containing approximately 1.2 mg of sample. A high vacuum cell equipped with cesium bromide windows was used to obtain a spectrum of 18:1-1 in a KBr pellet at liquid nitrogen temperature. An IR spectrum was obtained for each of the compounds from 2.5 to 40 μ (4000 to 250 cm^{-1}). Regions 4000 to 3700 cm^{-1}, 2700 to 1700 cm^{-1}, and 500 to 250 cm^{-1} of little or no absorption were not reproduced in the spectra shown.

Nuclear Magnetic Resonance Spectroscopy

A Varian (Varian Associates, Analytical Instrument Div., Palo Alto, Calif.) A-60 High Resolution Spectrometer was used to measure the spectra of the glyceryl ethers and related compounds. Unless otherwise noted, spectra were obtained with a 15% solution of each compound in carbon tetrachloride. All nuclear magnetic resonance spectra were measured at room temperature, except the 1- and 2-isomers of 14:0, and 16:0, and 18:0 glyceryl ethers, which were run at 50C. The single proton resonance peak of tetramethylsilane (TMS) used as an internal standard was assigned to value of zero parts per million (ppm) of the total magnetic field.

Thin-Layer Chromatography

Silica Gel G plates impregnated with approximately 10% sodium arsenite were prepared according to the procedure of Morris (24). Boric acid ($\sim$ 5.0%) impregnated Silica Gel G plates were prepared by the method of Thomas et al. (25). One part of Silica Gel G was slurried with two parts of water for the preparation of unimpregnated plates. Uniform 0.25mm and 1.0mm adsorbent layers were spread on 5 $\times$ 20 and 20 $\times$ 20 cm glass plates with a Colab No. 2810 applicator (Colab Laboratories, Inc., Chicago Heights, Ill.), modified in this laboratory (26). After the chromatoplates had air-dried for 30 min, they were activated in an oven for 30 min at 110C. The boric-acid-impregnated plates were activated 2 hr at the same temperature. The 1.0-mm thick preparative plates were used for the isolation and purification, while the 0.25-mm plates were used for analytical determinations. The saturated glyceryl ethers were separated from the long-chain alkyl halide by chromatography of the reaction mixture on 20- $\times$ 20-cm plates (1.0-mm thick adsorbent layer) with a hexane-diethyl ether-methanol, 80:20:5 (v/v/v) solvent system. Unsaturated glyceryl ethers were separated from the long-chain alcohols by chromatography of the reaction mixture on preparative plates developed in hexane-diethyl ether-methanol, 80:20:10 (v/v/v). The silver ion thin-layer chromatography (TLC) system recently reported (22) was used to check the glyceryl ethers for contamination by ethers of the same chain length, varying only in degree of unsaturation. Boric acid and sodium arsenite impregnated plates developed in chloroform-methanol, 98:2 (v/v) were used to check isomeric purity in addition to GLC analyses. Separations on analytical TLC plates were visualized by charring according to the procedure of Privett and Blank (27). The glyceryl ether region of the heavily loaded preparative plates was located visually, or more distinctly with the aid of UV light. The desired region was scraped from the plates and the glyceryl ethers were extracted with several volumes of diethyl ether.

Melting Point Determinations

Melting points were determined in triplicate for each compound. Samples were heated to 125C in the capillary melting point tubes and then quickly cooled in an ice bath to obtain only one polymorphic form in compounds capable of existing in more than one crystalline form.

EXPERIMENTAL

Synthesis of Saturated 1- and 2-Glyceryl Ethers

Saturated 1- and 2-glyceryl ethers ranging in hydrocarbon chain length from C_{10} to C_{18} were prepared from potassium salts of 1,2-isopropylidene glycerol and 1,3-benzylidene glycerol, respectively, by reacting with a twofold excess of alkyl iodides or bromides according

to the procedure of Davies et al. (4). The procedure was scaled down to prepare 300-600 mg of glyceryl ethers. The 1,3-benzylidene glycerol was prepared according to the procedure of Mattson and Volpenhein (28). The reaction mixtures containing the 1,2-isopropylidene glyceryl ethers or the 1,3-benzylidene glyceryl ethers were hydrolyzed, according to the procedure employed by Hartman (29) to remove these same blocking groups from monoglycerides. The glyceryl ethers were separated from the contaminating alkyl halides, hydrocarbons, etc., by preparative TLC (see TLC section). One- and 2-glyceryl ethers were further purified by crystallization from approximately 50 to 200 volumes of hexane, respectively, at –20C.

Synthesis of Unsaturated 1- and 2-Glyceryl Ethers

Mono- and diunsaturated C_{18} 1- and 2-glyceryl ethers were prepared from the potassium salts of 1,2-isopropylidene glycerol and 1.3-benzylidene glycerol, respectively, by reacting with the desired alkenyl-*p*-toluenesulfonate according to the procedure of Gupta and Kummerow (30). The alkenyl-*p*-toluenesulfonates were prepared by reacting *p*-toluenesulfonyl chloride with the unsaturated alcohols (6) and used without purification. Oleyl and linoleyl alcohols were prepared by reducing the corresponding methyl esters with lithium aluminum hydride (31). The blocking groups were hydrolyzed with boric acid. Contaminating alcohols, toluenesulfonates, etc., were separated from the saturated 1- and 2-glyceryl ethers by preparative TLC (see TLC section) and further purified by crystallization from hexane as described earlier.

Synthesis of n-Propyl and 3-Pentyl Octadecyl Ethers

Normal propyl and 3-pentyl octadecyl ethers were prepared to aid in the interpretation of the IR and NMR spectra of the isomeric glyceryl ethers. Synthesis was achieved by reacting the alkyl halide with the potassium salts of n-propyl and 3-pentyl alcohols, respectively. Approximately 0.5 g of 98% + pure (as determined by GLC) *n*-propyl octadecyl ether (mp 28.2-28.6C) and 0.5 g of 95% + pure 3-pentyl octadecyl ether (mp 13.0-14.7 C) were isolated by preparative GLC. The contaminant in each case was 1-iodooctadecane which did not interfere with the IR or NMR spectra.

Preparation of Glyceryl Ether Derivatives

Trifluoroacetate (TFA) and trimethylsilyl (TMS) ether derivatives of the isomeric glyceryl ethers were prepared according to the procedure recently described (22). The glyceryl ether diacetates were prepared by the method of O'Brien and Rouser (32). The last traces of pyridine were removed from the glyceryl ether TMS derivatives by preparative GLC.

Materials

The methyl esters used for the preparation of the alcohols were obtained from the Hormel Foundation, Austin, Minn. The 1,2-isopropylidene glycerol (2,2-dimethyl-1,3-dioxolane-4-methanol), 1-alkyl halides, *p*-toluenesulfonyl chloride, and trifluoroacetic anhydride were obtained from Eastman Organic Chemicals, Rochester, N. Y. Other materials were purchased as follows: Hexamethyldisilazane from Peninsular Chemical Research, Gainesville, Fla.; trimethylchlorosilane from K & K Laboratories, Plainview, N.Y.; and trimethyl borate from Matheson Coleman & Bell, East Rutherford, N. Y. Solvents and other reagents were reagent grade and used without further purification.

RESULTS AND DISCUSSION

Synthesis of Isomeric Glyceryl Ethers

The progress of the reactions was monitored by GLC analysis of samples removed intermittently from the reaction flask and injected directly into the chromatograph for each isomer. Using this technique we found that, on the average, overall yields of 50-60% of the 1- and 2-isomers could be obtained in 5-7 hr for both the saturated and unsaturated glyceryl ethers. Additional reaction time, up to 24 hr, usually did not increase the yield appreciably. Alkyl bromides and alkyl iodides were found to give equally high yields. Occasionally we observed substantial quantities of 1-alkenes (dehydrohalogenation products of 1-alkyl iodides) presumably caused by excess potassium.

Copper columns were found to cause an apparent decomposition of the alkyl iodides. Aluminum columns packed with the same material operating under identical conditions gave satisfactory results. It was not established whether the alkenyl-*p*-toluenesulfonates were eluted from the column intact or as a major breakdown product. The latter appeared unlikely since only one major peak was eluted for each alkenyl-*p*-toluenesulfonate without the leading front or tailing, usually associated with decomposition.

TABLE II
Melting Points and Purity of the Isomeric Glyceryl Ethers and Derivatives

Compound[a]	Melting point (°C)[b]	% Purity	Compound[a]	Melting point (°C)[b]	% Purity
10:0-1	34.5-36.0	97	18:1 (9c)-2	13-14	99+
10:0-2	30.4-31.5	97	18:2 (9c, 12c)-1	-4.5 to -4.0	95
12:0-1	47.2-48.0	99+	18:2 (9c, 12c)-2	-0.8 to 0	95+
12:0-2	45.0-46.5	99+	18:0-1 TFA	40.5-42.1	98
14:0-1	55.4-56.1 44.8-45.3[c]	98	18:0-2 TFA	25.2-26.2[c] 36.4-37.1	98
14:0-2	54.9-55.4	98	18:0-1 TMS	<20	99+
16:0-1	59.9-60.6	97	18:0-2 TMS	<20	99+
16:0-2	62.5-63.3	97	18:0-1 Diacetate	32.9-33.7	98
18:0-1	65.0-65.7	98	18:0-2 Diacetate	29.0-30.0	98
18:0-2	68.5-69.0	98+	n-propyl octadecyl	28.2-28.6	99+
18:1 (9c)-1	17.6-18.5	99+	3-pentyl octadecyl	13.9-14.7	95

[a] Shorthand system of nomenclature previously described (22).
[b] Mean range of three determinations.
[c] Polymorphic forms.

Purity of Glyceryl Ethers and Derivatives

Purity of the isomeric glyceryl ethers was determined by GLC analyses of their TFA derivatives on polar EGSS-X and nonpolar SE-30 columns (22) and by TLC on Silica Gel G layers impregnated with silver nitrate (22), sodium arsenite and boric acid, and on unimpregnated Silica Gel G. The purity for each of the ethers and derivatives prepared from them are given in Table II. All the ethers were greater than 99% pure isomerically and as a class, and more than 95% pure as a single identifiable component. The 1-isomers, of course, represented a racemic mixture. The impurities were glyceryl ethers with hydrocarbon chains two carbon atoms longer or shorter. The use of the alkenyl-*p*-toluenesulfonates produced no isomerization of double bonds detectable by IR in our synthetic procedures; the isomerization observed by Baer et al. (6) suggests that the sodium naphthalene used in their synthetic scheme caused the isomerization. Baer and Fisher (7) later found no isomerization using the tosyl-isopropylidene glycerol, oleyl alcohol, and sodium naphthalene.

The IR and NMR spectra and the melting points reported here also indicated that the glyceryl ethers and derivatives were quite pure. The melting points obtained are shown in Table II. Melting points obtained are on the average 4-5 degrees lower than those reported by Baumann and Mangold (33) for a similar homologous series of saturated 1-isomers. Melting points reported by numerous investigators (2,5,30,34-37) for the 18:0-1 and 16:0-1 ethers ranged from 68-72C and 59-64C, respectively. The 18:1-1 melting point was in close agreement with the 18-19C and 17.6-19C values reported by Baumann and Mangold (33), and Baer and Fisher (7), respectively. The 8C mp reported by Baumann and Mangold (33) for the 18:2-1 is 12 degrees higher than that reported here. The 18:0-2 melting point agreed well with that reported by Gupta and Kummerow (30), while those values reported by other investigators (4,10 and 36) are 6-8 degrees lower. The only reported melting point for the 16:0-2 agreed well with that found (4). The synthesis of the other 2-isomers: 18:1-2, 18:2-2, 14:0-2, 12:0-2, and 10:0-2 have not previously been reported.

The discrepancies in some cases between the observed melting points and values cited in the literature can be attributed to polymorphism and uncorrected melting points. The former can give rise to larger differences in melting points, as indicated with the 14:0-1 (see Table II), than if large quantities of impurities are present. Therefore, unless all the compounds are treated in a manner similar to that described in the Analytical Methods section, to obtain a known or reproducible polymorphic form, melting points are of little value. Interestingly, when a linear plot of the melting points of the saturated 1- and 2-glyceryl ethers (Table II) are plotted on the ordinate, versus the hydrocarbon chain length on the abscissa, two curves are obtained that intersect each other when the hydrocarbon chain lengths are 14 carbon atoms long. Initially, the 1-isomers have the higher melting points, but as the hydrocarbon chain increased in length, the melting points become lower than the 2-isomers.

The melting point obtained for the 18:0-1 diacetate agreed well with the 34-35C reported by Carter et al. (10); however, they reported

the 18:0-2 diacetate as a wax, but we obtained a definite melting point a few degrees below that of the 1-isomer. The 18:0-1 and 18:0-2 TMS ether derivatives were waxy and a definite melting point was not observed. Trifluoroacetate derivatives of the 18:0-1 and 18:0-2 had surprisingly high melting points, considering their GLC behavior, and two polymorphic forms were observed with the 2-isomer.

TLC of Isomeric Glyceryl Ethers

Sodium arsenite (A) and boric acid (B) impregnated Silica Gel G chromatoplates depicting the purity and resolution of 18:1-1 (1) and 18:1-2 (2) glyceryl ethers are shown in Figure 1. The 1-isomers of monoglycerides were found to migrate lower than the 2-isomers on boric acid impregnated silica gel layers by Thomas et al. (25); however, an explanation for the lower migration was not discussed. The reversal of the migration order on the two different impregnated adsorbent layers can be explained by assuming the glyceryl ether borate complex to be more polar than the uncomplexed form. The lower spots on each plate (Fig. 1) migrated to approximately the same position as both isomers on unimpregnated Silica Gel G plates, in the same solvent system. If we assume that arsenite and borate ions complex in a ratio of 1:1 with the glyceryl ethers [a ratio shown to exist by Roy et al. (38) with several polyols] and that the uncomplexed glyceryl ethers are more polar than the complexes (39), then the compounds on plate A lane 1 and plate B lane 2 represent 1,2-diol arsenite and 1,3-diol borate complexes, respectively. The latter, to the authors' knowledge, has not been reported to form a strong borate complex. Compounds on plate B (uncomplexed 2-isomers) with a higher R_f corresponds to the compounds on plate A (uncomplexed 2-isomers) with a lower R_f. Relatively higher R_f values on borate-impregnated plates compared to unimpregnated and sodium arsenite impregnated adsorbent layers developed in the same solvent system, have been reported by Morris (24). This is presumably caused by a reduction in the number of adsorption sites of the silica gel. Equations 1 and 2 are also consistent with this hypothesis.

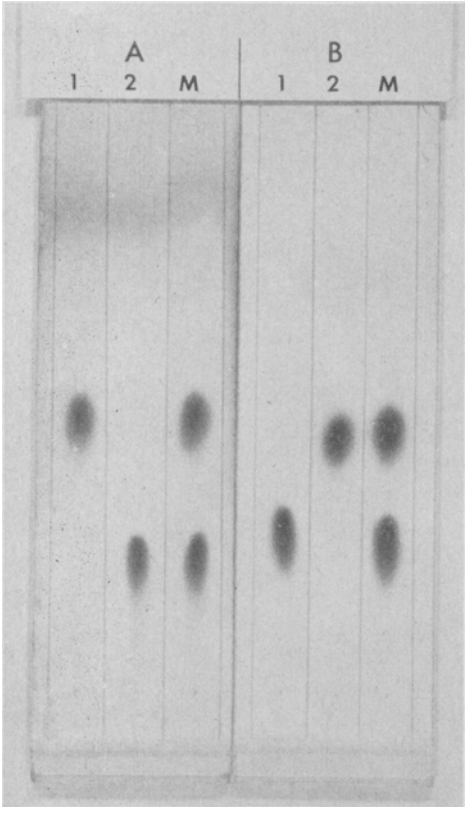
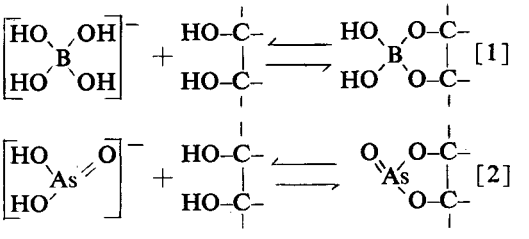


FIG. 1. TLC separation of 18:1-1 (1) and 18:1-2 (2) isomeric glyceryl ethers on Silica Gel G adsorbent layers impregnated with sodium arsenite (A) and boric acid (B).



A complex formed between a 1,2-diol and a borate ion by splitting out two molecules of water could give rise to a more polar complex than the original diol, as suggested by Roy et al. (38). This agrees with equation 1 and our TLC data. A complex formed between a 1,2-diol and a monohydrated arsenite ion by splitting out two molecules of water would be expected to be less polar than the original diol, which agrees with equation 2 and our TLC data. Such an arsenite ion could not be confirmed or excluded by the work of Roy et al. (38).

Comparison of Isomeric Glyceryl Ether IR Spectra

The IR spectra shown in Figure 2 were obtained from 13-mm diameter transparent KBr pellets. The first four spectra are included to

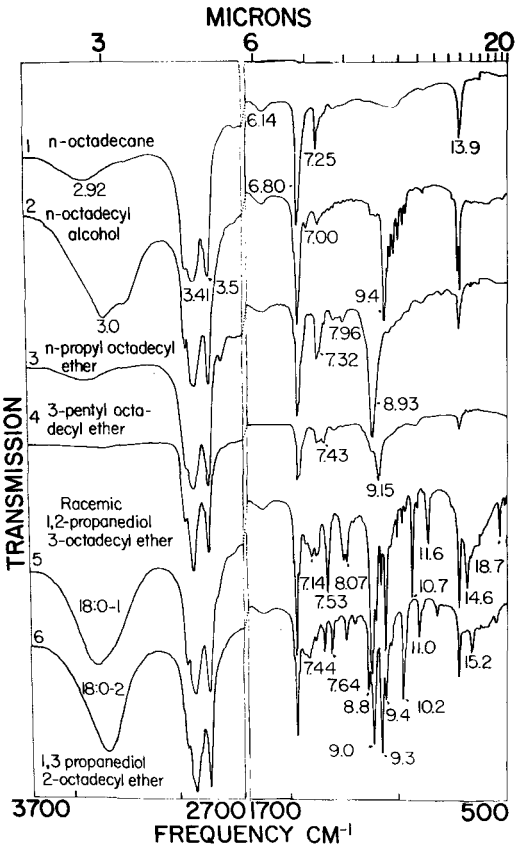


FIG. 2. IR spectra of 18:0-1 and 18:0-2 isomeric glyceryl ethers and compounds with individual functional groups found in the two isomeric ethers.

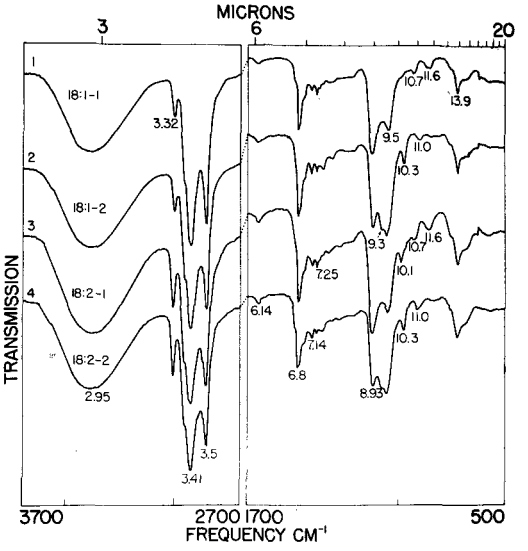


FIG. 3. Infrared spectra of long-chain synthetic mono- and diunsaturated 1- and 2-glyceryl monoethers.

aid in the interpretation of the last two isomeric glyceryl ether spectra. Absorption areas in the glyceryl ether spectra, due to the hydrocarbon portion of the molecule, are indicated by the n-octadecane spectrum, and similarly the primary alcohol, primary and secondary ether absorption areas are indicated by spectra 2, 3 and 4, respectively. The differences in absorption between primary and secondary ethers that are slightly shifted from 8.93 and 9.15 μ in the glyceryl ethers are shown by the *n*-propyl and 3-pentyl octadecyl ether spectra. As shown in Fig. 2 (5 and 6), the isomeric glyceryl ethers are easily distinguishable by other differences in absorption that are probably due to the presence of the secondary alcohol group in the 1-isomer (absent in the 2-isomer) that can exhibit intramolecular hydrogen bonding with the adjacent primary alcohol group. The spectrum of the 18:0-2 glyceryl ether was similar to that obtained by Debuch (40). The IR spectra obtained for the 10:0, 12:0, and 14:0 and 16:0 1- and 2-glyceryl ethers were similar to those of 18:0-1 and 18:0-2 (5 and 6) isomers shown in Fig. 2. The small absorption bands at 6.14 μ in all spectra and 2.92 μ in compounds 1, 3 and 4 were due to atmospheric water vapor and residual moisture in the ethers.

Spectra of the isomeric mono- and diunsaturated glyceryl ethers are shown in Figure 3. These, like the saturated 1- and 2-glyceryl ethers, are distinguishable by IR. The monounsaturates are only distinguishable from the diunsaturates by the degree of absorption in the region of 3.32 μ. Slight absorption at 10.7 and 11.6 μ in the 1-isomers differs from the weak 10.3 and 11.0 μ bands of the 2-isomers, and both isomers are further distinguishable by the stronger absorption at 9.3 and 9.5 μ of the latter. The 18:1-1 spectrum was not improved when the sample was analyzed as a KBr disc at ambient or liquid nitrogen temperatures. This was unexpected since Chapman (41) has shown that, generally, decreased resolution or increased absorption band smearing occurs with increased temperature; however, the converse relationship does not appear to hold. One possible explanation is that the dispersed glyceryl ether in the KBr disc may have little opportunity to rearrange its crystalline form.

IR Spectra of 18:0-1 and 18:0-2 Derivatives

Spectra of the 18:0-1 and 18:0-2 glyceryl ether diacetate, TFA and TMS derivatives are shown in Fig. 4. The isomeric diacetates differ by the stronger absorption in the region of 9.5 μ of the 2-isomer and the presence of the two weak absorption bands at 9.7 and 10.4 μ in the 1-isomer, absent in the 2-isomer. The contribution of the hydrocarbon chain to the spectra can be obtained by comparison with spectrum 1, Fig. 2. The slight absorption in the region of 2.9 μ is probably due to water vapor, since the 6.14 water band is also present. The ester carbonyl band at 5.7 μ, and assignments to other absorption bands have been made by Carter et al. (10).

The spectra of the two isomeric 18:0 glyceryl ether TFA derivatives are also shown in Fig. 4, and are distinctly different. However, the differences may be attributable to a different polymorphic form for each isomer or a mixture of the two observed forms of the 18:0-2 isomer. The carbonyl ester band shifted from 5.7 μ in the isomeric diacetates to 5.55 μ in the 1-isomer, and a doublet at 5.55 and 5.61 in the 2-isomer of the TFA derivatives.

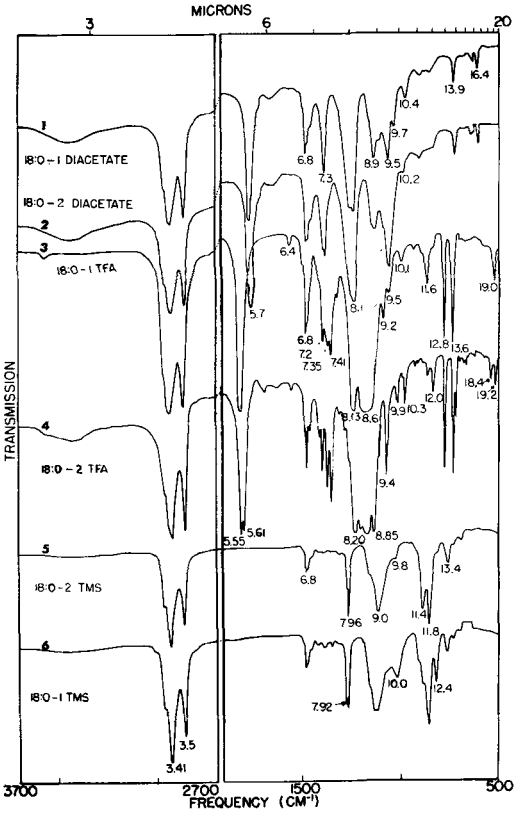


FIG. 4. Comparison of IR spectra of 18:0-1 and 18:0-2 glyceryl ether diacetate, TFA and TMS derivatives.

Spectra of the 18:0 isomeric glyceryl ether TMS ether derivatives are depicted at the bottom of Fig. 4. The broad ether absorption band in the region of 9.0 μ consisting of several observable overshadowed absorption bands, probably resulted from differences in absorption of primary and secondary silyl and alkyl ethers. The two isomers are distinguishable by the presence of two absorption bands at 10.0 and 12.4 μ in the 1-isomer, absent in the 2-isomer, and by the differences in degree of absorption at 11.4 and 11.8 μ of the two isomers.

Normal Propyl and 3-Pentyl Octadecyl Ether NMR Spectra

Normal propyl and 3-pentyl octadecyl ethers were synthesized to aid in the interpretation of the isomeric glyceryl ether spectra. The single hydrogen on carbon number 3 of the 3-pentyl octadecyl ether gave rise to a quintuplet at 3.02 ppm (central peak), which is partially overlapped by a triplet (central peak 3.32 ppm) arising from the two protons of the hydrocarbon chain adjacent to the ether oxygen. The resonance of the protons adjacent to the ether oxygen in the *n*-propyl octadecyl ether appear as two practically superimposable triplets at 3.33 ppm (central peak). The NMR spectrum of *n*-propyl hexadecyl or octadecyl ether has previously been obtained by Carter et al. (10); however, resolution was not sufficient to observe the two triplet sets for the methylene protons adjacent to the ether oxygen.

NMR Spectra of Isomeric Glyceryl Ethers

The NMR spectra of mono- and diunsaturated isomeric pairs, 18:1 and 18:2, and of two saturated glyceryl ethers, 12:0-1 and 16:0-2 are shown in Fig. 5. Spectra of the 1- and 2-isomers of all the glyceryl ethers analyzed were readily distinguishable and distinctly different. The partially visible broad-line resonance at approximately 3.75 ppm, equivalent to one proton, observed in the 1-isomers (absent in the 2-isomers) was assumed to be due to a downfield shift of the lone hydrogen on carbon atom number 2 of the glycerol moiety caused by the deshielding effect of the electronegative environment. Resonance of this same proton in the 2-isomers occurred upfield at approximately 3.42 ppm, which may or may not represent the central peak of a partially visible

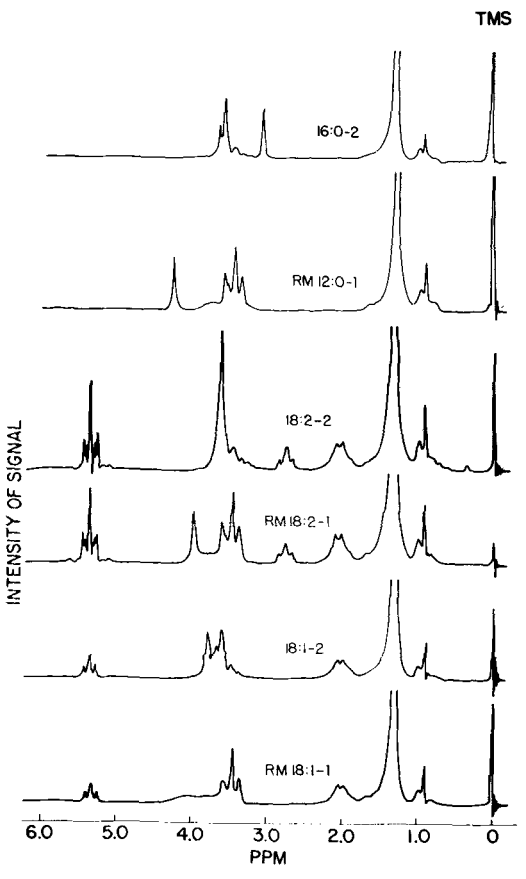


FIG. 5. Proton resonance spectra of saturated, mono-, and diunsaturated isomeric glyceryl ethers obtained at 60 mc.

multuplet (when not obscured by hydroxyl proton resonance).

The unresolved resonance at 3.65, 3.58, and 3.53 ppm of the 2-isomers differs markedly from the resonance shift upfield to 3.58, 3.53, 3.42, and 3.32 ppm in the 1-isomers. Resonance at 3.65 ppm in the 2-isomers, absent in the 1-isomers, is probably due to the methylene protons on carbon atoms 1 and 3 of the glycerol moiety. The spectra of the 2-isomers are further complicated by the triplet representing the methylene protons of the hydrocarbon chain adjacent to the ether oxygen. The spectra of the 1-isomers in the area of 3.3-3.7 ppm are equally complicated, and resonance-band assignments, other than relative expected order, are not made. The 3-glyceryl methylene hydrogens of the 1-isomers, like those of the 2-isomers, are expected to appear downfield, followed closely by a triplet and two doublets for the methylene hydrogens of the hydrocarbon chain and the nonequivalent methylene hydrogens on carbon number 1 of the glycerol moiety. Although specific assignments for this portion of the molecule were not possible, the usefulness of the NMR spectra is not limited.

The hydroxyl proton resonance, which is known to exhibit marked dependence on concentration and temperature (42) is shown in Table III for the isomeric glyceryl ethers. The hydroxyl proton resonance of the 1-isomers occurs at a lower field than the 2-isomers. Increased concentration of the 1-isomers also resulted in resonance of the hydroxyl protons at lower field. The broad resonance line, exhibited only by the 18:1-1 at two concentrations, is not understood.

Both isomers of the monounsaturated glyceryl ethers are easily distinguished from the diunsaturated by the resonance of the methylene hydrogens between the two double bonds at 2.72 ppm and by the intensity of the signal of the vinyl hydrogens at 5.31 ppm, as shown in Figure 5. In the unsaturated glyceryl ethers the hydrogens that are allyl to the double bond, which usually are obscured by methylene hydrogens adjacent to a carbonyl ester, are observed without interference at 2.04 ppm.

NMR Spectra of Glyceryl Ether Derivatives

The spectra of the 18:1-1 diacetate (top) and TMS ether (bottom) derivatives, obtained neat at room temperature, are shown in Figure 6. The purity and origin of the 18:1-1 glyceryl

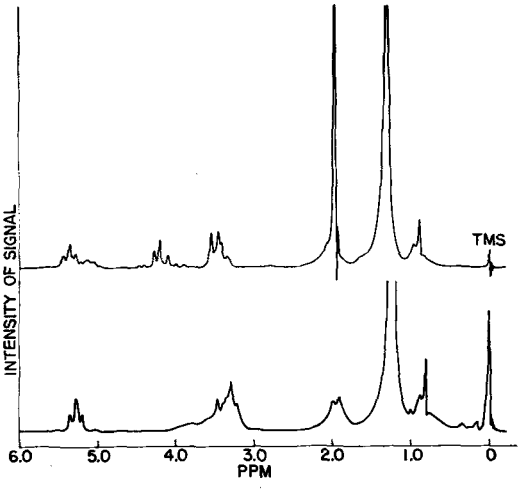


FIG. 6. NMR spectra of 18:1-1 glyceryl ether diacetate (top) and trimethylsilyl ether (bottom) derivatives obtained at 60 mc.

TABLE III
Chemical Shifts of Isomeric Glyceryl Monether Hydroxy Protons

Glyceryl ether	OH chemical shift (ppm)	Conc (%)	Temp
12:0-1	4.25	25	Room
12:0-2	3.43	10	Room
14:0-1	3.48	15	50C
14:0-2	2.92	15	50C
16:0-1	3.33	15	50C
16:0-2	3.08	15	50C
18:0-1	3.30	15	50C
18:0-2	2.92	15	50C
18:1-1	4.04[a]	15	Room
18:1-1[b]	4.48	Neat	Room
18:1-2	3.76	15	Room
18:2-1	3.94	15	Room
18:2-2	3.60	15	Room

[a] Broad band, see Fig. 6.

[b] Commercially available sample from Western Chemical Ind. Ltd., Vancouver, Canada, greater than 95% pure as a class (sterols main contaminant) and approximately 90% monoene 1-isomers.

ether is given at the bottom of Table III. The broad resonance band of the lone 2-glyceryl proton appears downfield at approximately 5.15 ppm in the diacetate and remains at 3.75 ppm in the TMS ether, identical to the free glyceryl ethers (Fig. 5). The two nonequivalent 3-glyceryl methylene hydrogens resonate at 4.18 ppm (central peak) as two partially overlapped doublets in the diacetate, and, as expected, these hydrogens appear unresolved from the other protons adjacent to the ether oxygens at 3.28 ppm (central peak) for the TMS ether. Resonance in the diacetate at 3.40 (central peak of triplet) and 3.50 ppm (center of doublet) arises from the hydrogens adjacent to the ether oxygen. The two doublets of the nonequivalent 1-glyceryl methylene hydrogens were superimposed. Aside from the acetate methyl proton resonance at 1.95 ppm, all resonance bands for the two derivatives are the same as previously discussed. The NMR spectra of the 18:0-1 and 18:0-2 glyceryl ethers and monoglyceride diacetates have previously been obtained at 40 mc by Carter et al. (10), and assignments have been made. Resolution was poor, but sufficient to demonstrate the 2-glyceryl ether diacetate (not determined here) spectrum distinguishable from that of the 1-isomer.

The IR and NMR spectra of the long-chain isomeric 1- and 2-glyceryl monoethers and derivatives, both saturated and unsaturated, have served to demonstrate the applicability of these two types of spectroscopy for the characterization and structural determination, in addition to distinguishing between the two isomeric forms.

ADDENDUM

While this manuscript was being reviewed for publication, a paper by Serdarevich and Carroll (43) appeared dealing with the physical and chemical properties of two isomeric pairs of anteiso glyceryl ethers and the 16:0 isomers. Their findings are compatible with those reported here.

ACKNOWLEDGMENT

NMR and IR spectra obtained by Miss Lucy Scroogie and P. E. Staats, Oak Ridge National Laboratory (ORNL).

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cis-5,*cis*-9,*cis*-12-Octadecatrienoic and Some Unusual Oxygenated Acids in *Xeranthemum annuum* Seed Oil[1]

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ABSTRACT

Seed oil of *Xeranthemum annuum* (family Compositae) contains a number of unusual fatty acids in addition to palmitic, stearic, oleic, linoleic and linolenic. These acids include *cis*-5,*cis*-9,*cis*-12-octadecatrienoic, 5%; *cis*-9-L,10-L-epoxyoctadecanoic, 3%; *cis*-9-L,10-L-epoxy-*cis*-12-octadecenoic (coronaric), 8%; and *cis*-12-D,13-D-epoxy-*cis*-9-octadecenoic (vernolic), 2%; as well as a mixture of two hydroxy acids, 11%. The absolute configurations of the two 9,10-epoxy acids are established for the first time.

INTRODUCTION

XERANTHEMUM ANNUUM L., one of the oldest and best known of the "everlastings," has been widely cultivated as an ornamental. Examination of the seed oil at this laboratory has revealed significant amounts of unusual constituents. For example, Durbetaki titration gave a hydrogen bromide equivalent (HBE) (1) at 55C which indicated approximately 23% C_{18} epoxy acids or other HBr reactive acids (2). Gas-liquid chromatographic (GLC) analyses of the methyl esters disclosed an unknown component having equivalent chain lengths (3) of 19.2 on a Resoflex-446 column and 17.5 on an Apiezon-L column. These values did not correspond to those of any common fatty acid. In addition, other unknown GLC peaks were apparently due to oxygenated materials or to their decomposition products. This paper describes the isolation of the unusual fatty acids of *X. annuum* seed oil and the characterization of some of these.

PROCEDURE AND DATA

UV spectra were determined with a Beckman DK-2A spectrophotometer; IR spectra, on either a Perkin-Elmer Model 137 or 337 instrument as liquid films or as 1% solutions in carbon tetrachloride or carbon disulfide. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian A-60 spectrometer in deuteriochloroform solutions containing 1% tetramethylsilane. Optical rotatory dispersion (ORD) measurements were made on a Cary Model 60 recording spectropolarimeter at 25C in 0.5 dm cells. GLC analyses were conducted as described by Miwa (3). TLC was performed on glass plates covered with 0.25 mm films of Silica Gel G or 25% silver nitrate-impregnated Silica Gel G and with appropriate solvent systems as described by Morris (4). Melting points were determined with a Fisher-Johns block and are uncorrected.

Preparation of Methyl Esters

Oil was extracted from the ground seed, 98 g, of *X. annuum* in a Soxhlet with petroleum ether (bp 30-60C). Solvent was removed from the dark green extract on a rotary evaporator to give 21.5 g of oil. The oil had an HBE of 23, and titration of the sample with base demonstrated the presence of 17% free fatty acid, calculated as oleic acid. Spectral characteristics included maxima at 2.81μ in the IR, indicative of OH, as well as at 10.14 and 10.50μ, indicative of a *cis,trans*-conjugated diene. The latter assignment was supported by a UV maximum at 234 mμ ($E^{1\%}_{1\,cm}$ 103).

The oil was treated with an ethereal solution of diazomethane until neutral. After GLC analysis for fatty acid composition, the product, 18.5 g, was then transesterified by refluxing it for 1 hr with 200 ml of methanol containing 0.8 g of dissolved sodium. The mixture was cooled, acidified with acetic acid, diluted with 400 ml of water and extracted repeatedly with ethyl ether. After the extracts were dried over sodium sulfate, the solvent was removed to give 17.7 g of mixed methyl esters. GLC analyses of the diazomethane-treated oil, with conditions under which only methyl esters derived from free fatty acids were analyzed, were nearly identical to GLC analyses of the total methyl esters after transesterification (see Table I). Evidently the free acids in the oil were representative of the total fatty acids

[1] Presented in part at the AOCS Meeting in Cincinnati, October 1965.

[2] No. Utiliz. Res. Dev. Div., ARS, USDA.

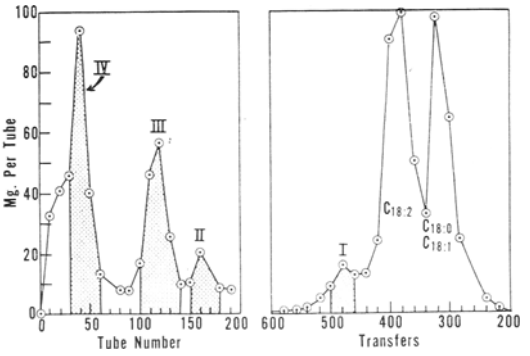

FIG. 1. Countercurrent distribution of *Xeranthemum annuum* methyl esters. The labelled peaks represent: I, *cis*-5,*cis*-9,*cis*-12-octadecatrienoate; II, *cis*-9,10-epoxyoctadecanoate; III, coronarate; IV, esters of hydroxy acids.

present, both free and combined, and no preferential hydrolysis of glycerides had occurred. Analyses of the total methyl esters by TLC revealed numerous components, which included at least two epoxy and two monohydroxy esters.

Countercurrent Distribution

Methyl esters of *Xeranthemum* were subjected to countercurrent distribution (CCD) in a 200-tube Craig-Post apparatus with an acetonitrile-hexane solvent system (5). A 16.7 g sample of the mixed methyl esters dissolved in 320 ml of lower phase was equally distributed among the first eight tubes of the apparatus and 40 ml of lower phase was added to each of the remaining tubes. We then made 600 transfers using 10 ml of upper phase per transfer and the single withdrawal procedure.

For the first 200 transfers long settling times (up to 20 min per transfer) were used because of problems with emulsions. Combined material from the first 440 transfers was shown by GLC analyses to be mostly $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ methyl esters. Percentages of various esters isolated by CCD are compared in Table I with percentages obtained by GLC. Transfers 460–500 were combined to give 0.53 g of an unknown ester (I) (Fig. 1). The more polar material, which remained in the instrument, gave rise to the following appropriately combined fractions: II (0.5 g), combined esters isolated from tubes 150–180; III (1.5 g), combined esters isolated from tubes 100–140; IV (1.6 g), combined esters isolated from tubes 30–60.

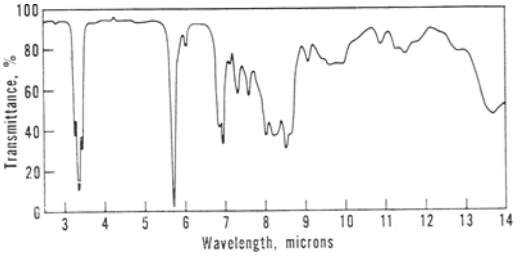

FIG. 2. Infrared spectrum (neat) of methyl *cis*-5,*cis*-9,*cis*-12-octadecatrienoate (Fraction I).

Characterization of Fraction I

Fraction I (99% pure by GLC) was an unknown, which had a rather distinctive infrared spectrum (Fig. 2) and migration characteristics similar to those of methyl linolenate when subjected to TLC on ordinary silica and on silver nitrate-impregnated silica plates. Since there was no absorption in the infrared spectrum at 10.3 μ, all double bonds present must be *cis*. Both the NMR spectrum (Fig. 3) and the IR spectrum of this ester were easily distinguished from the spectra of other known fatty esters examined. However, both were quite similar to the corresponding spectra of methyl *cis*–5, *cis*–11, *cis*–14–eicosatrienoate (6). Integration of the NMR spectrum of I showed the equivalent of six vinyl protons (4.4–4.8τ) and two protons (triplet at 7.22τ) typical of methylene-interrupted double bond systems. Hydrogenation of I gave methyl stearate, mp 37.5–38.0C, with the uptake of 3 moles of hydrogen.

The double bond positions in I were proved unequivocally by the following procedure: A 0.2 g sample of I was partially reduced by heating at 50C, with 17 ml of ethanol containing 0.5 ml of hydrazine hydrate. Air was

TABLE I

Total Fatty Acid Composition of *Xeranthemum* Seed Oil

Apparent acid	Equivalent chain length methyl ester R-446	Ap-L	Area % by GLC[a]	Wt. % from CCD[b]
C16:0	16.0	16.0	11	9
C18:0	18.0	18.0	4	3
C18:1	18.4	17.8	20	16
C18:2	18.9	17.7	49	36
C18:3	19.7	17.7	1	1
I, unknown	19.2	17.5	6	5
II, C18:0 epoxy	22.7	19.6	Trace	3
III, C18:1 epoxy	23.1	19.0	5	10
IV, C18:2 hydroxy	...	...	...	11
Totals			96	94

[a] Analyses are presented as total methyl esters obtained by transesterification.

[b] The remaining 6% was unidentified material. This estimate represents our best determinations of the fatty acid composition of *Xeranthemum* oil.

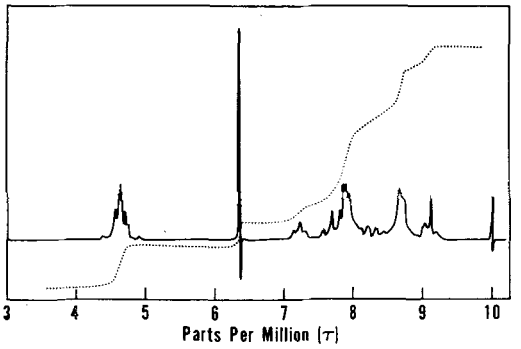

FIG. 3. Nuclear magnetic resonance spectrum of methyl *cis*-5,*cis*-9,*cis*-12-octadecatrienoate (Fraction I). Integral curve is dotted.

bubbled through the mixture for 17 hr. The reaction mixture was then diluted with 20 ml of water and neutralized with dilute hydrochloric acid. Products were recovered by repeated ether extractions and the combined ether extracts dried over sodium sulfate. This procedure yielded 0.2 g of product shown to have eight components by GLC (stearate, three monoenes, three dienes and unreacted I). Similar partial hydrogenation of methyl linolenate has been demonstrated to proceed randomly and with no positional or geometric isomerization of the double bonds (7).

A fraction (60 mg) containing only the three *cis*-monoenes formed during the reduction was isolated by chromatography on a 25% silver nitrate-impregnated silica column (13 mm × 14 cm) with hexane-benzene (3:2) as the eluting solvent. A 54-mg portion of this monoene mixture was cleaved oxidatively by the procedure of von Rudloff (8). GLC of the cleavage products as free acids gave hexanoic (19%), nonanoic (27%) and tridecanoic (51%) acids as the monobasic fragments. Methyl esters were then prepared by treating the cleavage products with diazomethane, and the mixture was again analyzed by GLC. This procedure showed nearly equal percentages of pentanedioic, nonanedioic and dodecanedioic acids as the only dibasic cleavage fragments.

Characterization of Fraction II

Fraction II was shown to be an epoxystearate (98%) by GLC analyses and by Durbetaki titration (HBE = 99). The IR spectrum and the TLC retention characteristics of II were identical with those of known methyl *cis*–9,10–epoxyoctadecanoate. Fraction II, mp 20.0–22.5C, was dextrorotatory in methanol, $[\alpha]_{589}$ + 0.3°.

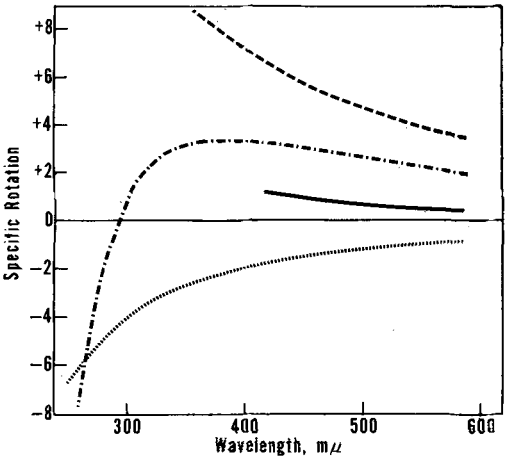

FIG. 4. Optical rotary dispersion of Fraction II, methyl 9,10-epoxyoctadecanoate (———); Fraction III, methyl coronarate and methyl vernolate 4:1 (-------); methyl 12-D,13-D-epoxy-*cis*-9-octadecenoate (vernolate) from *Vernonia* (—.—.—); methyl 12-D,13-D-epoxyoctadecanoate prepared from (+)-methyl vernolate (.....). All measurements were made in hexane solution.

A portion of II was passed through a short silica column, and then crystallized from hexane to remove traces of colored materials. This sample of II, mp 22.0–23.5C, gave a plain positive ORD curve in the range 600–400 mμ; $[\alpha]_{589}$ + 0.48, $[\alpha]_{450}$ + 0.96 (c. 4.15, hexane) (Fig. 4).

A 52-mg portion of II was subjected to acetolysis by the procedure of Gunstone (9). Saponification of the acetolysis product gave 49 mg of a dihydroxy acid, mp 92.0–93.5C. No depression of the melting point was observed upon admixture of this material and known *threo*–9,10–dihydroxyoctadecanoic acid, mp 93.0–94.0C. The dihydroxy acid was cleaved with permanganate-periodate reagent (8). GLC analyses of the resulting products, as methyl esters, showed that the cleavage fragments were nonanoic (45%) and nonanedioic (46%) acids.

Characterization of Fraction III

By GLC analyses and by Durbetaki titration (HBE = 99), fraction III was identified as an epoxyoleate (96%). The IR spectrum of III was similar to that of known methyl vernolate (*cis*–12,13–epoxy–9–octadecenoate). TLC of III and of methyl vernolate on silica demonstrated that III was not homogeneous and that the major component of III had an R_f slightly lower than that of methyl vernolate. A portion of III was further purified by column chromatography on silica followed by

crystallization from hexane. The resulting sample gave a plain positive ORD curve in the range 600–350 mμ; $[\alpha]_{589}$ + 3.44, $[\alpha]_{350}$ + 8.94 (c. 9.54, hexane) (Fig. 4).

A 181–mg portion of III was subjected to acetolysis and then saponified according to Gunstone's procedure (9). This treatment gave 177 mg of a dihydroxy-monoene acid. A part of this crude product was subsequently cleaved with permanganate-periodate (8) to fix the positions of the ethylenic and vicinal diol groupings. The cleavage products, as determined by GLC of the methyl esters, were hexanoic (14%) and nonanedioic (74%) acids.

A second part of the dihydroxy-monoene acid, obtained after acetolysis of III (124 mg), was hydrogenated (0.9 mole H_2) to give dihydroxystearic acid, mp 90.0–92.5C. The melting point was not raised by repeated crystallization from mixtures of chloroform-hexane. Examination of the products after permanganate-periodate cleavage of this material, including that recovered from the mother liquors, showed the dihydroxystearic acid to be a mixture of 9,10– and 12,13–isomers in a ratio of about 4:1. The cleavage products identified by GLC of the methyl esters were hexanoic (5%), nonanoic (29%), nonanedioic (41%) and dodecanedioic (10%) acids. The melting point of the saturated dihydroxy acid mixture (90.0–92.5C) was closely reproduced by using a mixture of known *threo*–9,10–dihydroxyoctadecanoic and *threo*–12,13–dihydroxyoctadecanoic acids in the same proportions (4:1).

Optical Rotatory Dispersion

Fractions II and III, as previously mentioned, gave plain positive ORD curves (Fig. 4). Hydrogenation of a portion of fraction III was accomplished by the technique of Brown et al. (10) as modified by Miwa et al. (11). This procedure, which does not alter epoxides (12), provided a mixture of epoxyoctadecanoates, mp 25.0–25.5C, which also showed a plain positive ORD curve in the 600–350 mμ range; $[\alpha]_{589}$ + 0.19, $[\alpha]_{350}$ + 1.03° (c. 1.36, hexane).

For comparison, methyl 12–D,13–D–epoxy–*cis*–9–octadecenoate (13) from *Vernonia anthelmintica*, $[\alpha]_{589}$ + 1.99, $[\alpha]_{260}$–7.52° (c. 3.55, hexane), was similarly hydrogenated to give methyl 12–D,13–D–epoxyoctadecanoate (12), mp 30.0–31.0C; $[\alpha]_{589}$–0.85, $[\alpha]_{250}$ –6.79° (c. 4.78, hexane) (Fig. 4).

A sample of methyl 9–D–hydroxyoctadecanoate, mp 52.5–53.5C, $[\alpha]_{589}$–0.22, $[\alpha]_{250}$–1.65° (c. 2.51, methanol), was obtained by hydrogenation of methyl dimorphecolate, a naturally occurring hydroxy acid known to have the D configuration (14–16). This saturated ester was subsequently converted to 1,9–D–octadecanediol, mp 79.1–79.8C, $[\alpha]_{589}$–0.14, $[\alpha]_{250}$ –0.44° (c. 2.28, methanol), by reduction with lithium aluminum hydride (LAH). LAH reduction of the *Xeranthemum* epoxyoctadecanoate (II) gave a 1:1 mixture of 1,9– and 1,10–octadecanediols, mp 73.0–74.2C; $[\alpha]_{589}$ + 0.11, $[\alpha]_{250}$ + 0.44° (c. 2.02, methanol). The ORD curves of these three samples are given in Fig. 5A.

A sample of methyl 12–D–hydroxyoctadecanoate (hydrogenated methyl ricinoleate), mp 56.0–57.0C, $[\alpha]_{589}$–0.37, $[\alpha]_{250}$–2.32° (c. 2.42, methanol) (15–17), was reduced with LAH to give 1,12–D–octadecanediol, mp 79.3–79.8C; $[\alpha]_{589}$–0.43, $[\alpha]_{250}$–2.97° (c. 2.10, methanol). LAH reduction of methyl 12–D,-13–D–epoxyoctadecanoate gave a 1:1 mixture of 1,12–D– and 1,13–D–octadecanediols, mp 74.0–75.0C; $[\alpha]_{589}$–0.36, $[\alpha]_{250}$–2.98° (c. 2.56, methanol). The ORD curves of these three samples are given in Fig. 5B.

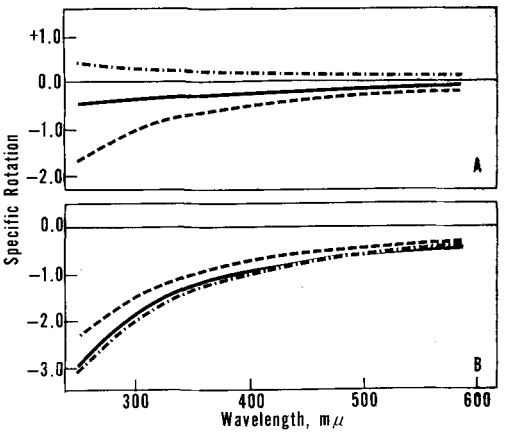

FIG. 5. A. Optical rotatory dispersion of methyl 9-D-hydroxyoctadecanoate (-----), 1,9-D-octadecanediol (————), and the mixture of 1,9- and 1,10-octadecanediols obtained by LAH reduction of methyl 9,10-epoxyoctadecanoate from *Xeranthemum* (—.—.—). B. Optical rotatory dispersion of methyl 12-D-hydroxyoctadecanoate (-----), 1,12-D-octadecanediol (————), and the mixture of 1,12-D- and 1,13-D-octadecanediols obtained by LAH reduction of methyl 12-D,13-D-epoxyoctadecanoate (—.—.—). All measurements are considered correct to within ±0.04° and were made in methanol solution. All these materials gave satisfactory C, H analyses and were homogeneous by GLC.

Characterization of Fraction IV

Fraction IV had IR maxima at 2.76 (OH), 10.19 and 10.54 μ (*cis,trans* conjugated diene). A single maximum in the UV at 234 mμ, $E_1^{1\%}{}_{cm}$ 822 (cyclohexane), also indicated *cis,trans* conjugated diene. TLC of fraction IV on an ordinary silica plate showed two spots judged to be of equal intensity. The R_f values of these spots correspond closely to those of methyl 9–hydroxy–10,12–octadecadienoate and methyl 13–hydroxy–9,11–octadecadienoate under conditions described by Morris and Wharry (18).

DISCUSSION

Hydrogenation of fraction I demonstrated that it had a normal C_{18} carbon skeleton and had the equivalent of three double bonds. The NMR and IR spectra of I, as well as its TLC migration characteristics, are consistent with a structure having two *cis* double bonds separated by one methylene group and another *cis* double bond separated from the other two by more than one methylene group. Partial hydrazine reduction of I, followed by oxidative cleavage of the resulting *cis*-monoenes, placed the double bonds at the 5, 9 and 12 positions. Thus I can only be methyl *cis*–5,*cis*–9,*cis*–12–octadecatrienoate.

cis–5,*cis*–9,*cis*–12–Octadecatrienoic acid has previously been reported in tall oils and in various extracts of pine and birch (19,20). The presence of this acid in *Xeranthemum* (a Compositae) suggests that its occurrence in plants may be even more widespread. Other fatty acids having isolated double bonds at the 5 position (both *cis* and *trans*) have recently been discovered in a wide variety of plant sources (6,21–29). The biochemical significance of these Δ^5 unsaturated acids is not yet understood.

The experimental data on fraction II clearly demonstrate that it is methyl *cis*–9,10–epoxyoctadecanoate. The observation that II was dextrorotatory in methanol indicates that it is the same optical isomer found by Tulloch (30) in *Lycopodium* spores and in wheat stem rust spores. Fraction III is clearly a 4:1 mixture of methyl *cis*–9,10–epoxy–12–octadecenoate (coronarate) and methyl *cis*-12,13-epoxy-9-octadecenoate (vernolate).

To determine the absolute configuration of (+)–methyl *cis*–9,10–epoxyoctadecanoate, it was necessary to compare it to a suitable reference compound of known absolute configuration. For this comparison, 9–D–hydroxyoctadecanoic acid, which was synthesized by Baker and Gunstone (14) and shown to be levorotatory by Schroepfer and Bloch (16), seemed most appropriate. LAH reduction of a D,D–epoxyoctadecanoate gives rise to a 1:1 mixture of octadecanediols (31), both of which necessarily are D because configuration is retained by the oxygen function (13,32).

We have demonstrated that the 1,9–D– and 1,12–D–octadecanediols are both levorotatory, and we can safely predict that the 1,9– to 1,16–octadecanediols of D configuration are all levorotatory (33). As the mixture of 1,9– and 1,10–octadecanediols from LAH reduction of (+)–methyl *cis*–9,10–epoxyoctadecanoate is dextrorotatory (Fig. 5), we can only conclude that they both have the L configuration. Therefore, (+)–methyl *cis*–9,10–epoxyoctadecanoate has the 9–L,10–L configuration or the (9*R*,10*S*) (34). Since II and methyl 12–D, 13–D–epoxyoctadecanoate have rotations of opposite sign, evidently they have opposite configurations. As hydrogenation of fraction III gave a 4:1 mixture of 9,10– and 12,13–epoxy esters and since the mixture was dextrorotatory, (+)–methyl–*cis*–9,10–epoxy–*cis*–12–octadecenoate must also have the 9-L,10-L (9*R*, 10*S*) configuration. The 1:1 mixture of 1,12– and 1,13–octadecanediols, obtained by reduction of (+)–vernolic acid, and known 1,12–D–octadecanediol are all levorotatory. Thus, we can also conclude that (+)–vernolic acid has the 12–D,13–D (12*S*,13*R*) configuration. This assignment is in complete agreement with the earlier findings of Morris and Wharry (13) although our approach involves a different reaction sequence.

Epoxy acids occur commonly as constituents of seed oils. Although only one epoxy acid is found in some oils (9,35–38), in others two or more are present (39). All the naturally occurring epoxy acids appear to be optically active and the best known of these, vernolic, exists in both (+) and (–) forms (13,40).

The *Xeranthemum* dienols are currently under further investigation in this Laboratory and will be the subject of a future paper.

ACKNOWLEDGMENT

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Fatty Acid Composition of Milk Phospholipids. II. Sheep, Indian Buffalo and Human Milks[1]

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ABSTRACT

Phospholipids were isolated from sheep, Indian buffalo, and human milks, and their fatty acid compositions determined by gas chromatography. The specific distributions of fatty acids in phosphatidyl cholines (PC) and phosphatidyl ethanolamines (PE) were determined after phospholipase A hydrolysis.

Fatty acid compositions and specific distributions were similar in sheep and buffalo milk phospholipids, and compared closely with those of bovine milk. Human milk phospholipids, particularly PE, contained much larger amounts of polyunsaturated acids, but negligible amounts of branched-chain acids. Palmitic and oleic acids were evenly distributed in human milk PC and PE, whereas they were preferentially located in the α' position in PC and PE of ruminant milks.

The results are discussed in the context of current theories of lipid biosynthesis.

INTRODUCTION

ALTHOUGH THE PHOSPHOLIPIDS of bovine milk have been studied extensively in the past (1,2), new methods of analysis reveal additional facts concerning these important lipids. It has been demonstrated that several classes of phospholipids and glycolipids are present and we have recently determined the composition and distribution of fatty acids in the principal classes (3). However, comparatively little is known about the phospholipids of milk from other species and most of the available data have been estimated from lipid phosphorus values. The composition of milks from several different species is given in Table I. These limited data suggest that milk lipids from the human and horse contain more phospholipids than milk lipids from ruminants. Within individual species, Baliga and Basu (4) found that the phospholipid content of cow, sheep and goat milks increased with increasing fat content, but did not change in buffalo milk.

The proportions of the various phospholipids in milks were studied by Baliga and Basu (4) who reported ca. 30% lecithin, ca. 25% sphingomyelin, and ca. 45% cephalin (by difference) in the milks of cow, sheep, goat and Indian buffalo. Rawat has reported similar figures for cow, sheep, buffalo and goat milks (5) and Nagasawa et al. (6) have also given similar figures for cow and human milks. Our own phospholipid analyses by two-dimensional thin-layer chromatography (TLC) are of the same order, although more detailed, and will be published elsewhere in due course.

We are not aware of any published work on the composition and distribution of fatty acids in the various phospholipids of milks other than bovine milk. Kudo et al. (7) determined the fatty acid composition of the total phos-

TABLE I

Proximate Composition of Some Milks from Different Species

Species	Total solids[a]	Total lipids[a]	Phospholipids in 100 g milk	Phospholipids in total lipids
	%	%	mg	%
Cow	12.7	3.7	34[b], 38[c,d]	0.87[b], 0.80[c]
Sheep	16.3	5.3	43[d], 58[e]	0.81[d], 0.74[e]
Indian buffalo	17.1	7.5	21[e], 37[g]	0.31[e], 0.54[g]
Human	12.4	3.8	60[f]	1.42[f]
Goat	13.4	4.1	37[d], 50[e]	0.73[d], 1.03[e]
Pig	21.2	9.6	79[d]	0.82[d]
Camel	13.4	3.1	—	—
Horse	11.0	1.6	26[d]	1.65[d]

Note: All constituents vary considerably with such factors as age, breed, diet, stage of lactation, number of lactations, analytical methods, etc. Only average values representative of each species are listed.

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[1] Part I of this series is "Fatty Acids of Bovine Milk Glycolipids and Phospholipids, and Their Specific Distribution in the Diacylglycerophospholipids," W. R. Morrison, E. L. Jack and L. M. Smith. JAOCS, *42*, 1142-1147 (1965).

pholipids of human milk, but no attempt was made to examine the fatty acid composition of each phospholipid class. The work reported in the present paper is part of a comparative study of the composition and distribution of fatty acids in the triglycerides (TG) and major phospholipids of milks from several species (3,8).

EXPERIMENTAL

Sheep milk was obtained from six Suffolk ewes (University of California, Davis), pooled, freeze-dried, and stored under nitrogen at –20C. Spray-dried Indian buffalo milk was supplied in nitrogen-packed cans by C. B. Shah, Institute of Agriculture, Anand, India. Human milk was obtained by courtesy of P. M. Peacock, Glasgow Royal Maternity Hospital, and individual batches were freeze-dried and stored at –20C. Average analyses of several batches of human milk phospholipids were comparable to those expected from replicate analyses of pooled samples.

The procedures used to extract and isolate the lipids were as previously described, (3) and the analytical procedures were substantially unchanged. Fatty acid methyl esters were analyzed by GLC on diethylene glycol succinate and Apiezon L columns (3). More recently it was found more satisfactory to hydrolyze phosphatidyl ethanolamine (PE) with snake venom phospholipase A in 2:4:6-collidine/HCl buffer (9). The hydrolysis products were recovered by adding $CHCl_3$ and MeOH to convert the hydrolysate to a monophasic Bligh and Dyer (10) extraction medium, which was then partitioned in the usual manner.

RESULTS AND DISCUSSION

The phosphatidyl serine (PS) fraction from each species included phosphatidyl inositol (PI) because the preparative TLC technique used (3) did not separate these two phospholipid classes. Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and sphingomyelin (Sph) were examined by two-dimensional TLC and by infrared spectral analysis and were found to be free from impurities.

TABLE II

Specific Distribution of Fatty Acids (mole %) in Milk Phosphatidyl Cholines

	Sheep			Indian buffalo			Human		
Fatty acid[a]	α′	β	total	α′	β	total	α′	β	total
12:0	0.7	0.4	0.5	0.3	0.5	0.4			
*i*13:0	0.3		0.4	0.1					
13:0	0.1	0.4		0.1	0.1	0.2			
*i*14:0	0.6		0.1	0.2	0.2	0.1			
14:0	4.0	5.4	4.6	3.7	7.1	5.7	3.4	4.9	4.5
14:1				0.3	0.7	0.3			
*i*15:0	0.3	0.4	0.3	0.2	1.3	0.7			
*ai*15:0	0.3	0.2	0.2						
15:0	1.5	0.8	1.3	1.3	1.4	1.3			
15:1	0.3		0.1						
*i*16:0	0.3	0.7	0.4	0.4	0.8	0.4			
16:0	42.5	32.0	38.2	34.9	19.2	27.7	34.2	32.3	33.7
16:1	0.7	0.3	0.5	1.5	3.2	2.1	1.5	2.2	1.7
*i*17:0	0.5	1.4	0.7						
*ai*17:0	0.9	1.6	1.2						
17:0	1.1	0.4	1.1	0.5	0.5	0.6			
17:1	0.7	0.1	0.3						
*i*18:0	0.2	0.2	0.2	0.3	0.5	0.3			
18:0	16.9	4.7	10.6	24.8	6.2	15.6	43.9	2.1	23.1
18:1	19.2	35.0	26.6	26.7	42.1	35.0	14.3	13.7	14.0
18:2	2.9	6.7	4.3	2.0	9.9	5.7	2.7	30.9	15.6
*tt*18:2	0.3	0.7	0.6						
18:3	1.0	3.4	2.8	0.4	2.0	0.6		2.0	1.3
*ttt*18:3	1.0	1.8	1.7						
19:0				0.7	0.5				
20:0	0.4	0.4		0.4	0.4	0.4			
20:1						0.4			
21:0				0.2	0.2	0.2			
20:3		0.3	0.3			0.4		3.9	2.1
20:4		0.6	0.3		1.5	0.7		6.6	3.3
22:4								0.6	0.3
22:6								0.8	0.4
Unknowns	3.3	2.1	2.7	1.0	1.7	1.2			
Total saturated	70.6	49.0	59.8	68.1	38.9	53.6	81.5	39.3	61.3

[a] *i* = iso; *ai* = anteiso; *tt* = trans, trans; *ttt* = trans, trans, trans.

TABLE III
Specific Distribution of Fatty Acids (mole %) in Milk Phosphatidyl Ethanolamines

	Sheep			Indian buffalo			Human		
Fatty acid	α′	β	total	α′	β	total	α′	β	total
12:0	0.4	0.4	0.4	0.3	0.2	0.7			
*i*13:0					0.1				
13:0				0.1	0.2	0.1			
*i*14:0				0.1		0.1			
14:0	1.9	2.0	1.6	1.1	0.8	1.4	1.0	1.0	1.1
14:1				0.1	0.2	0.1			
*i*15:0	0.2	0.3	0.2	0.1		0.1			
*ai*15:0	0.1	0.2	0.2						
15:0	0.6	0.6	0.5	0.3	0.2	0.3			
*i*16:0	0.2	0.2	0.1	0.5	0.4	0.1			
16:0	20.1	9.4	14.9	15.2	5.0	10.4	9.3	8.2	8.5
16:1		1.2		1.6	3.0	2.0	1.8	3.3	2.4
*i*17:0	0.5	0.3	0.2						
*ai*17:0	1.2	1.3	0.7						
17:0	1.4	0.4	1.0	1.0	0.3	0.6			
17:1				0.6					
*i*18:0	0.2		0.1	0.3	0.6	0.5			
18:0	21.6	3.8	13.2	30.2	4.7	19.7	65.4	1.3	29.1
18:1	44.4	60.4	52.2	43.3	47.5	43.1	18.1	15.3	15.8
18:2	2.8	9.8	7.3	2.4	26.6	13.9	4.4	30.2	17.7
18:3		5.0	2.4	0.4	3.9	1.9		5.1	4.1
*ttt*18:3		0.7	0.9						
19:0				0.7	0.6	0.6			
20:0	0.2	0.3	0.2	0.5	0.3	0.3			
21:0				0.4	0.8	0.7			
20:3								5.4	3.4
20:4		1.1	1.0	0.3	2.2	0.9		20.9	12.5
22:4								4.1	2.8
22:6								5.2	2.6
Unknowns	4.2	2.6	2.9	0.5	2.4	2.5			
Total saturated	48.6	19.2	33.3	50.8	14.2	35.6	75.7	10.5	38.7

The fatty acid compositions of PC, PE, and PS + PI are given in Tables II, III, and IV. The amounts of each of the major fatty acids in the sheep and Indian buffalo are in reasonable agreement with those of bovine milk (3) if allowance is made for variations in feed. In both ruminant and human milks, the PE contains higher proportions of unsaturated fatty acids than does PC, and the PS + PI fraction is intermediate between PE and PC in total unsaturated fatty acids. The fatty acid compositions of human milk PC, PE, and PS + PI are simpler than for the corresponding phospholipids from ruminant milks whose fatty acids are derived in part from activities of the rumen microorganisms.

The fatty acid composition of sphingomyelin (Sph) from sheep, buffalo, and human milks is given in Table V. The fatty acids are typically saturated or monounsaturated, and the Sph of ruminant milks contains characteristically large amounts of 23:0, and significant amounts of 22:0 and 24:0. The general pattern of fatty acids in Sph from sheep and buffalo milks compares fairly well with that of bovine milk Sph. The fatty acids of human milk Sph differ in that there are roughly equal amounts of 16:0, 18:0, 22:0 and 24:0 with lesser amounts of the corresponding monoenes. There is some 23:0 present, but it is not a major component.

It is apparent from Tables II-V and from our previous studies (3, 11) that the composition and specific distribution of fatty acids in phospholipids from ruminant milks are similar. Ruminant milks contain significant amounts of branched-chain and odd-numbered fatty acids, whereas these acids are essentially absent in human milk phospholipids.

Tables II and III also show the distribution of each fatty acid between the α′- and β-positions of PC and PE. The trends for these milk phospholipids are similar to those reported for PC and PE from other sources (12), namely, the saturated fatty acids are located preferentially at the α′-position and the unsaturated at the β-position. Human milk PC and PE are, however, exceptional in their almost even distribution of 16:0 and 18:1 between the α′- and β-positions.

According to present concepts, the biosynthesis of TG and diacylglycerophospholipids involves a common D-α, β-diglyceride precursor (13). The positional distribution of fatty acids in TG compared to that in PC and PE from milks of the same species is therefore of in-

TABLE IV

Fatty Acid Composition (mole %) of Milk Phosphatidyl Serines + Phosphatidyl Inositols Fraction

Fatty acid	Sheep	Indian buffalo	Human
12:0	0.7	0.5	
13:0	0.2	0.3	
*i*14:0	0.2	0.2	
14:0	1.9	2.1	2.5
14:1	0.4	0.2	
*i*15:0		0.3	
15:0	0.6	0.4	
*i*16:0	0.7	0.3	
16:0	16.9	12.3	15.9
16:1	1.9	1.7	1.2
17:0	0.9	0.5	
*i*18:0		0.6	
18:0	20.5	20.4	39.7
18:1	36.8	39.4	13.0
18:2	8.2	8.9	17.4
*tt*18:2		0.9	
18:3	4.2	1.8	1.5
19:0	1.4	0.9	
20:0	1.6	0.8	
20:3		1.2	3.2
20:4	1.9	1.2	3.4
22:4			1.4
22:6			0.8
Unknowns	1.0	5.1	
Total saturated	45.6	39.6	58.1

terest. The relative distribution of a particular fatty acid between the β-position and the terminal positions of TG can be determined by hydrolyzing the TG with pancreatic lipase which specifically cleaves fatty acids from the α- and α'-positions. Similar information for

TABLE V

Fatty Acid Composition (mole %) of Milk Sphingomyelins

Fatty acid	Sheep	Indian buffalo	Human
12:0	0.3	0.7	
13:0		0.3	
*i*14:0		0.5	
14:0	2.5	1.4	2.0
14:1		0.2	
15:0	0.4	1.0	0.1
*i*16:0	0.1	0.4	
16:0	22.5	17.3	12.8
16:1	1.0	0.6	0.6
17:0	1.1	0.6	0.5
17:1			0.3
*i*18:0	0.2	0.4	
18:0	8.1	2.3	11.8
18:1	6.2	0.7	1.0
18:2	0.5		0.3
19:0			0.4
20:0	0.5	1.0	8.9
20:1	0.7		0.5
21:0	1.0	1.1	0.8
22:0	7.5	17.4	19.5
22:1			1.6
23:0	27.2	31.4	4.0
23:1	1.2		
24:0	17.0	20.7	19.5
24:1	2.0		15.4
Unknowns		2.0	
Total saturated	88.4	96.5	80.3

TABLE VI

Comparison of the Proportion (%) of Each Major Fatty Acid Esterified at the β-position in TG, PC, and PE of Milk Fats of Different Species.

Species	Fatty acid	Proportion of fatty acid at β position TG[a]	PC[b]	PE[b]
Bovine[a]	16:0	39	42	18
	18:0	17	16	8
	18:1	24	57	51
	18:2	15	81	87
	20:4	—	86	87
Sheep	16:0	29	42	32
	18:0	25	22	26
	18:1	36	64	46
	18:2	36	72	74
	20:4	—	83	78
Indian buffalo	16:0	37	36	28
	18:0	24	20	18
	18:1	33	61	53
	18:2	28	82	94
	20:4	—	100	91
Human	16:0	74	48	47
	18:0	8	5	2
	18:1	12	49	46
	18:2	17	95	87
	20:4	—	100	84
	22:4	—	100	73
	22:6	—	100	100

[a] TG data from Freeman et al. (8); bovine PC and PE results calculated from data of Morrison et al. (3).

[b] Calculated from (FA/2PL) × 100 = proportion (%) of fatty acid type esterified at the β-position, where FA = mole % of the acid hydrolyzed by phospholipase A and PL = mole % of the acid in the original diacylglycerophospholipid.

diacylglycerophospholipids can be obtained by phospholipase A hydrolysis. The proportion (%) of each major fatty acid esterified at the β-position of TG, PC, and PE from ruminant and human milks is compared in Table VI. Within each species, the distribution patterns for PC and PE are essentially similar but both patterns differ from that of the TG. Significantly smaller proportions of the 18:1 and 18:2 acids are located at the β-position in the TG, and C_{20} and C_{22} polyunsaturated acids are absent. Comparison of the results for the various species shows that the patterns for each lipid class (TG, PC, PE) are generally similar for the ruminants, whereas each of the lipids of human milk has a greater proportion of 16:0 and a smaller proportion of 18:0 at the β-position than the ruminants. Thus it is evident that there are differences in the distribution of fatty acids at the β-position between TG and diacylglycerophospholipids of milks from both ruminants and humans.

However, these results are not necessarily inconsistent with the concept of a common diglyceride precursor, but confirm, rather, the complex nature of lipid biosynthetic pathways. There are some indications of the means where-

by phospholipid fatty acids may become characteristically differentiated. Weiss et al. (14) have suggested that the enzymes catalyzing the synthesis of TG and phospholipids from diglycerides may be specific for diglycerides of different fatty acid composition. It has also been suggested that phospholipids formed initially may be subject to phospholipase A hydrolysis which is specific for certain fatty acids (15), and the lysophospholipids could then be reacylated by specific acyltransferases (16). Lands et al. (17) have recently shown that acyltransferase activity is very much influenced by the location and configuration of double bonds in fatty acids. There is at present no information available on the nature and distribution of fatty acid isomers in milk phospholipids, but we hope to obtain such information in future work.

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Ultracentrifugal Isolation of Serum Chylomicron-Containing Fractions with Quantitation by Infrared Spectrometry and NCH Elemental Analysis

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ABSTRACT

An ultracentrifugal method for isolating chylomicron-containing fractions from serum by flotation, using either standard Spinco swinging-bucket rotors or a specially fabricated swinging-bucket rotor, is described. Lower limits of the S_f rates of the chylomicron fractions are evaluated using a computer technique to define lipoprotein flotation over a nonlinear NaCl density gradient. The latter is prepared by a special overlayering technique.

Quantitation within a 0-50 μg region of mass assay is accomplished by both infrared spectrometry and elemental analysis for N, C and H. Results indicate that the chylomicron concentration in serum for a small population of nonfasting male adults ranges from approximately 0-50 mg %.

INTRODUCTION

SINCE THE DISCOVERY over 40 years ago of the chylomicrons, by Gage and Fish (1), the importance of this class of chyle and serum lipoprotein particle to states of fat absorption and transport has been increasingly recognized. Efforts to isolate and quantitate the chylomicrons have included the original procedure of dark field counting of individual particles (1), estimations by turbidimetric procedures (2), flocculation by chemical procedures including toluidine blue (3) and polyvinyl-pyrrolidone (4), and by numerous combinations of ultracentrifugal isolation (5-8) and mass assay. Another fractionation procedure has utilized starch block electrophoresis in combination with preparative ultracentrifugation (9). Also, swinging-bucket procedures, including the use of both sucrose (10) and salt (11) gradients, have been used in an effort to characterize the particle-size distributions of the chylomicron spectra. However, all of these techniques, although very useful for specific experimental applications, have limitations and are difficult to reproduce from one laboratory to another.

Stated quite simply, the two basic problems of chylomicron analysis are to define unambiguously the isolation procedure and to provide for an accurate and reproducible mass assay. Although the preparative ultracentrifuge has been widely used to isolate chylomicron fractions, there is little agreement among workers as to what conditions should be used. As recommended by Dole and Hamlin (7), it is desirable to express the fractionation in terms of the product of mean relative centrifugal force and time ($g \times$ minutes) as well as to define the full conditions of preparative ultracentrifugation. Depending upon many factors such as the temperature of fractionation, the solvent or solution density and whether an angle or swinging-bucket type rotor is used, a wide range in ultracentrifugation expressed in terms of $g \times$ minutes has been reported (6). Thus, values of from 0.1-3.0 $\times$ 10^6 $g \times$ minutes have been used at centrifugal fields of from 9,500 $\times$ g to 100,000 $\times$ g.

A rigorous definition of the chylomicra is difficult, particularly if fractions are isolated from plasma or serum as contrasted with chyle. Also, with ultracentrifugal procedures there is potentially the complication of including some endogenous large very low-density lipoproteins in any chylomicron fractionation procedure. Therefore, it appears advantageous at this time to define a chylomicron-containing fraction isolated ultracentrifugally only in terms of its included S_f range. The following procedure, utilizing a nonlinear salt gradient in a swinging-bucket rotor, is presented for isolating a S_f 400-10^5 fraction from human sera. With modifications only in the centrifugal conditions, this procedure may be used for defining a fraction with any desired lower S_f limit with relatively small differences between anticipated threshold and 100% recovery (as defined below).

With the exception of the peak period of fat absorption in the normal (1, 12) as well as in certain pathological states of hyperlipemia (13-15), the chylomicron class of lipoproteins exists at relatively low abundance. Therefore, the quantitation of chylomicrons from 1 ml of sera

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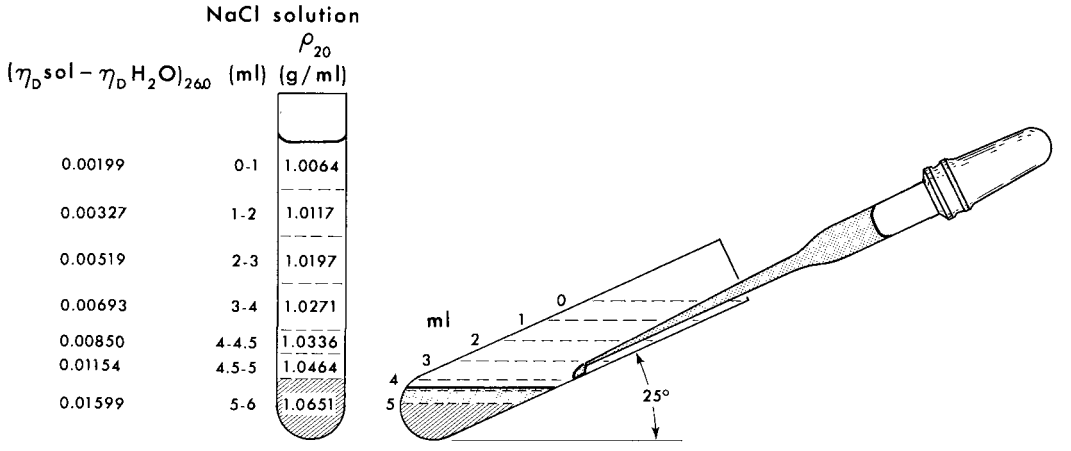


FIG. 1. Over-layering procedure for preparing nonlinear salt gradient. Successive 0.5 and 1 ml layerings, drop by drop over 1 ml salt solution or serum.

becomes a microanalytical problem. We have developed two independent procedures for this quantitation, one based on infrared spectrophotometry and the other on elemental analysis for nitrogen, carbon and hydrogen (NCH). In either method, an aliquot of the top fraction of the density-gradient preparation may be used directly.

EXPERIMENTAL

Serum and NaCl Gradient Preparation

Dry sodium chloride in the amount of 84.4 mg was weighed into a screw-cap vial and 1.00 ml of serum was added. The salt was dissolved by swirling the vial to adjust the solution density to 1.063 g/ml. Exactly 1.00 ml of this solution was placed in the bottom of a 0.5 × 2.5 in. cellulose nitrate ultracentrifuge tube. This tube was placed in a plastic holder, tilted to a position 25° from horizontal, and was illuminated by a beam of light from the side.

Precise volumes of the sodium chloride solutions tabulated in Fig. 1 were added above the serum layer dropwise from a Pasteur pipette (inside bore of capillary 0.4 – 0.6 mm) in the order of decreasing densities, forming a gradient from the bottom to the top of the tube. The pipette tip was placed in the tilted tube so that the droplets fell upon the tube wall just above the surface of the solution. After the gradient had been formed, the tube was returned to the vertical position, capped with a standard ½ in. Spinco tube cap (stainless steel plunger), and was placed in a four-place swinging-bucket rotor (16). Opposing buckets were balanced with lead shot within 15 mg. Alternatively, a 25.1 or a 25.3 Spinco type rotor equipped with 6 ml tube adapters may be used.

Refractive increments at 26.0C were determined with an Abbe precision refractometer for indirect measurement of solution densities and determination of changes occurring during gradient formation and ultracentrifugation. Serum adjusted with NaCl to density 1.063 g/ml (excluding macromolecular contribution to density) was placed at the bottom of the tube, except in control studies when a NaCl solution was used. The disodium salt of ethylenediamine tetraacetic acid, normally used in lipoprotein work, was omitted because it would contribute an unreasonably high background for the elemental analysis.

Ultracentrifugation was carried out in a Model L preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) equipped with a vacuum sentinel (17). Rotor speeds of from 11,000 to 13,000 rpm were employed at 19-20C chamber temperature for a period (approximately 2.5 hr at 12,000 rpm for the 4-place swinging-bucket rotor) calculated to bring all lipoproteins of S_f greater than 400 into the top 1.0 ml of solution. In all fractionations, a value of $2.26 \pm 0.10 \times 10^6$ $g \times$ min was used. Because of the difficulty in controlling and measuring accurately low rotor speeds, a mean rpm was calculated after approximately 2.25 hr up-to-speed (UTS). On the basis of this speed continuing for the remaining period, a shut-off time was calculated to yield 2.26×10^6 $g \times$ min of ultracentrifugation. Included in this calculation was a value of 3.27 min UTS equivalent, corresponding

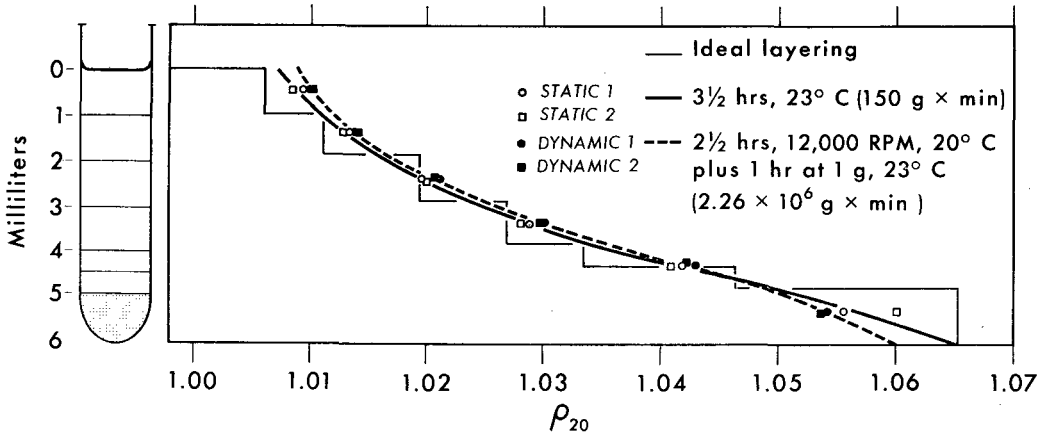
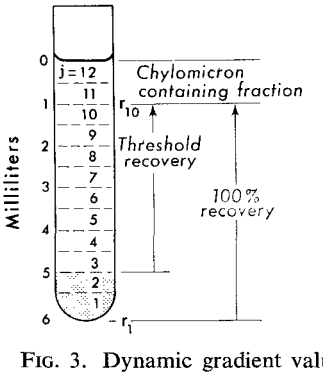


FIG. 2. Comparison of static and dynamic gradient used in chylomicron isolation. NaCl gradient stability, 6 ml preparative tube.

to a total of 9.80 min acceleration to full speed and deceleration to 1,000 rpm.

After the centrifugation was completed, the tubes were placed vertically in the holder and the top chylomicron-containing fraction was transferred to a 1.0 ml volumetric tube with a capillary pipette according to the method of de Lalla and Gofman (18). The second and third 1.0 ml fractions were removed as salt background solutions.

Stability of the salt gradient was evaluated by refractometry of all 6 ml fractions in the tube. Very little difference was observed between a gradient pipetted immediately after preparation and one allowed to stand at 1 *g* for 2.5-3.5 hr. Fig. 2 shows the static and dynamic reproducibility of the gradient after 3½ hr at 1 *g* and 14,100 *g* (12,000 rpm), respectively. Accordingly, we have used a mean of the dynamic gradient values (see Fig. 3) as a basis for calculating lipoprotein recovery in terms of S_f values in our chylomicron-containing fractions.



ρ_{20} (g/ml)	η_{20} (cp)
1.0097	1.0303
1.0112	1.0337
1.0130	1.0379
1.0155	1.0437
1.0187	1.0513
1.0233	1.0625
1.0277	1.0735
1.0328	1.0865
1.0392	1.1034
1.0464	1.1230
1.0739	2.0018
1.0789	2.0169

FIG. 3. Dynamic gradient values of ρ and η for lipoprotein flotation calculation within each 0.5 ml region.

Calculation of Lipoprotein Recovery

Fig. 3 shows a 6-ml preparative tube divided into 12 regions containing a dynamic salt gradient. To simplify the calculations, each of these j regions may be considered a homogeneous region of ρ_j and η_j. If ultracentrifugation proceeds for a given $g \times$ min, and a top 1 ml fraction is taken, lipoprotein recovery may be defined in terms of 100% and threshold S_f values. Ideally, no lipoproteins of S_f value lower than the threshold value should be included in the top 1 ml fraction. Using an IBM 7094 computer, a table of recovery values is calculated for a sequence of times from 100 to 20000 sec at various rpm values. For example, the 100% recovery calculation considers as a first approximation the flotation rate

$$F = (\ln r_{11} - \ln r_1)/\omega^2 \Sigma \Delta T_{UTS}.$$

Then assuming a mean ρ, η and $\sigma_o = 0.93$ g/ml,

$$S_{f_1} \cong F \times 1.44 \quad \text{and} \quad \sigma_1 = f\ (\ln S_{f_1})$$ from

literature values (8,16). Starting an interative calculation with $i = 1$, F_j is calculated over each region j from:

$$S_{f_i} = F_j\,(\rho_s - \sigma_i)\ \eta_j/\eta_s\ (\rho_j - \sigma_i).$$

Then Δt_j and hence $(\Sigma_1^{10} \Delta t_j)_i$ may be calculated from

$$F_j = \Delta \ln r_j/\omega^2 \Delta t_j.$$

A new approximation is now made setting

$$S_{f(i+1)} = S_{f_j}\ (\Sigma_1^{10} \Delta t_j / \Sigma \Delta T_{UTS})$$

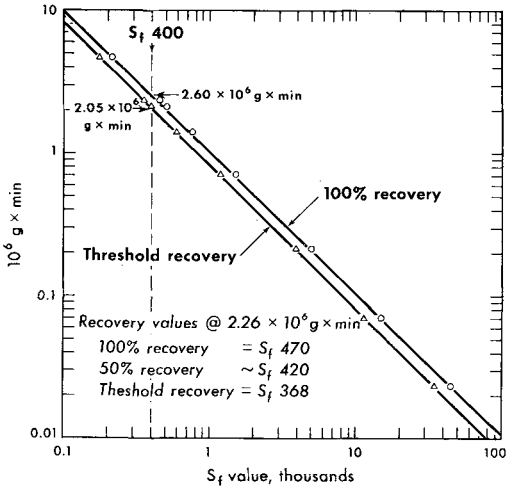


FIG. 4. Lipoprotein recovery over the range of 0.01 - 10 × 10^6 g × min.

and one continues to iterate for convergence until

$$|(\Sigma_1^{10}\Delta t_j)_n - (\Sigma\Delta T_{UTS})|/\Sigma\Delta T_{UTS} < 0.001$$

Fig. 4 shows a plot of calculated lipoprotein recovery over the range of 0.01–10 × 10^6 g × min. As can be seen, a discrepancy in flotation rate of approximately 20% exists between the threshold and 100% recovery values for any given ultracentrifugal fractionation. This overlap may be minimized further by reducing the layered serum volume and by using a longer radial flotation path. Effectively, one can increase the radial path by taking a smaller top fraction (ideally only the skim). However, the present procedure represents a practical compromise in relation to other requirements of the analysis.

Quantitation by NCH Elemental Analysis

Approximately 100 mg (95-115 mg) of each chylomicron fraction (approximately 5-6 drops) was placed one drop at a time in a large aluminum boat (Coleman type 29-410) and the weight was taken after 20 sec. All samples were done in duplicate and each batch contained a duplicate salt background as a matrix control. The boats were transferred to a stainless steel holder (capacity 10 boats), covered with a petri dish to minimize dust contamination and dried on a 55C aluminum plate overnight for 14 hr in a dust-proof glove box. The dried samples were stored in a desiccator prior to analysis.

Elemental analysis was performed using a NCH analyzer (F&M Model 185, Hewlett-Packard Co., Avondale, Pa.) equipped with a HP-18 helium purifier and modified to permit

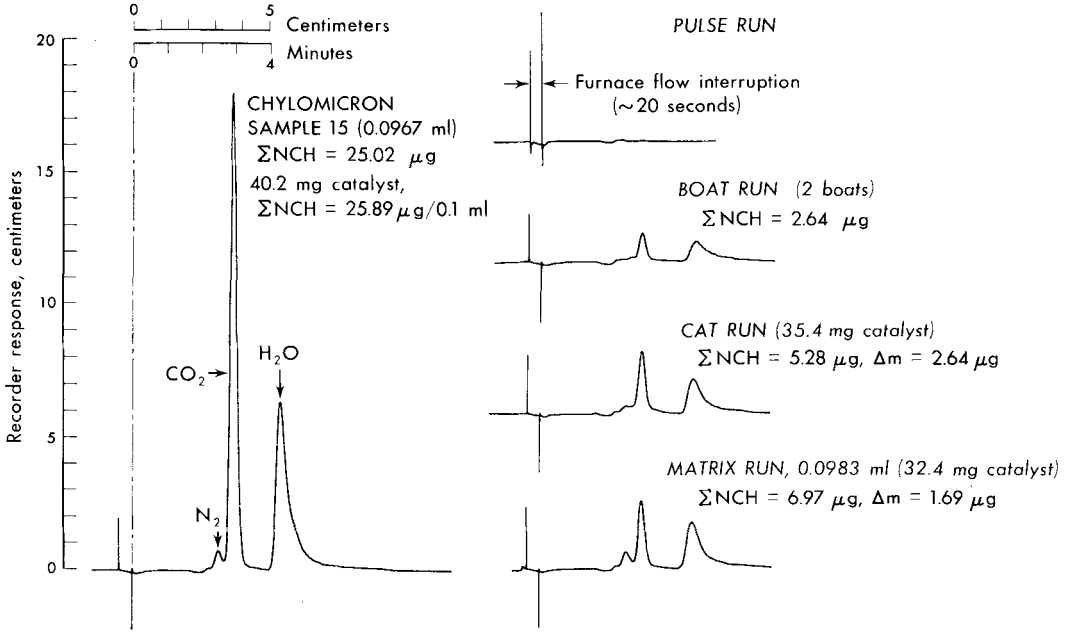


FIG. 5. Typical NCH chromatograms showing chylomicron sample and contribution to background of injection rod and aluminum boats, catalyst, and matrix.

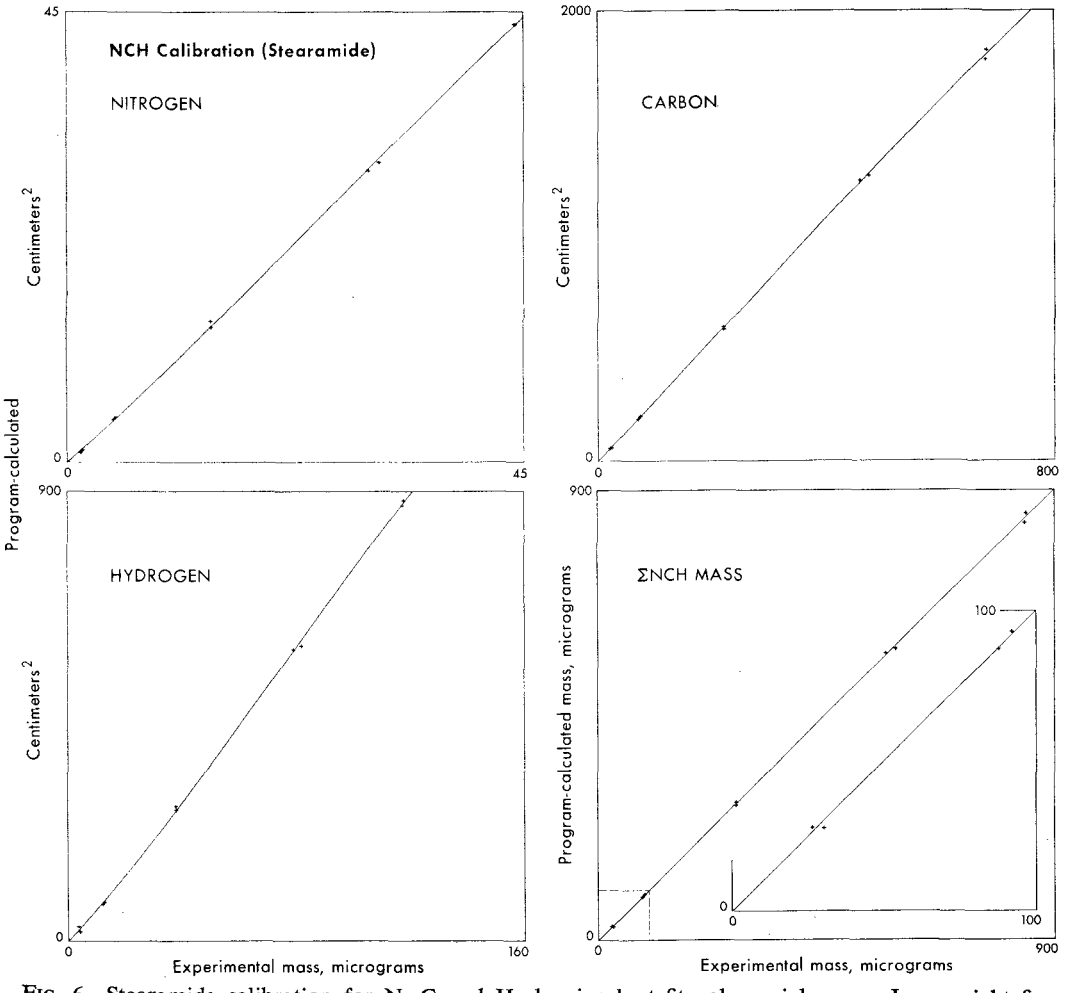


FIG. 6. Stearamide calibration for N, C and H showing best fit polynomial curve. Lower right figure compares program calculated total NCH mass with experimental mass.

continuous stability at maximum sensitivity. These modifications included a voltage regulator for all components, a special foot switch to activate an exact furnace-flow time delay (20.0 sec) the instant the injection rod reached its terminal position, a cycling dual unit water-glycerol humidifier and the substitution of more sensitive potentiometers for recorder-zero adjustment and bridge current. Usual operation of the modified instrument without attenuation gave minimal baseline drift (see Fig. 5). After conditioning the injection rods, the sequence of duplicate analyses was as follows: 2 empty boats (an F&M 5517.007 boat was used as a cover to prevent sample loss during manipulation and injection), 2 boats plus 35-40 mg catalyst, matrix salt background, chylomicron samples and absolute mass calibration (600-650 μg stearamide). Periodically an absolute mass calibration was performed over the range of 20-900 μg permitting nonlinear calibration corrections for all components. Fig. 6 shows such a calibration and a plot of the total NCH mass calculated against the weighed value, illustrating near linearity over the usual range of sample mass. Quantitation of the chromatograms was accomplished by manual measurement of peak heights and effective elution times (see Fig. 5), which together with other information on the sample and run conditions were evaluated by a computer technique described in some detail elsewhere (19).

Infrared Analysis

The infrared spectrophotometric method for quantitation of the isolated $S_f > 400$ lipoproteins makes use of the fact that the major con-

stituent (about 85-90% by weight) of these macromolecules is triglyceride. The infrared absorption by ester carbonyl groups of the triglycerides at 1742 cm^{-1} (5.74 μ) provides a convenient measure of this lipid class. Cholesteryl esters and phospholipids are present in relatively small amounts, and it is estimated that their contribution to the peak carbonyl absorption is no more than about 5% of the total. This can be taken into account in the calibration procedure. (See Fig. 7).

Sample preparation is done by the pressed pellet method, taking advantage of the circumstance that the top ml obtained in the density gradient centrifugation contains the isolated lipoproteins in a known concentration of sodium chloride. An aliquot (equivalent to 5-6 mg NaCl) of the top milliliter is lyophilized, and the resulting powder is pressed into a solid disk in a Perkin-Elmer microdie. The disk is 1.5 mm in diameter, approximately 1 mm thick, and contains 3.5 to 4.5 mg of salt. For this size disk we have used a reflecting 6:1 beam condenser on the spectrophotometer. Both the spectrophotometer (Model 421) and the auxilliary beam condenser are Perkin-Elmer instruments. Since the transmittance of the beam condenser is only about 30%, its use necessitates a variable attenuator in the reference beam. Slit widths are doubled with respect to normal.

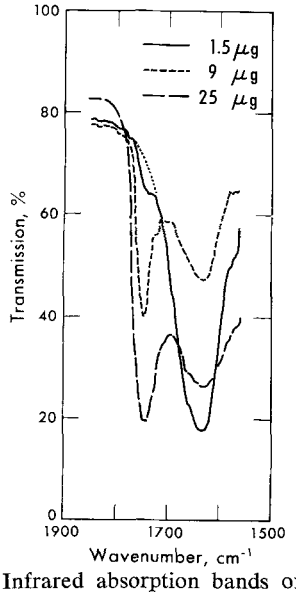


FIG. 7. Infrared absorption bands of triglycerides (1742 cm^{-1}) and residual water (1640 cm^{-1}) in pellets made from lyophilized chylomicron preparations. The dotted line (....) is a sketched-in water background for the solid curve.

Calibrations have been carried out by three different procedures, with reasonably good agreement among them. In the first procedure a highly concentrated preparation of chylomicrons is analyzed by both elemental and infrared methods. This is then used as a stock solution for serial dilution down to the level of about 1 μg/ml. Dilutions are made with NaCl solution of the same concentration as the ultracentrifugal top fraction. From the resulting series, chylomicron concentrations below 50 μg/ml are used for infrared standardization. 0.4 ml of each solution is lyophilized, and a pellet is prepared from the resulting powder. The pellet is formed in a hole in the center of a tared stainless steel disk, and the weight of the pellet is readily obtained. Since it is not possible to reproduce precisely the amount of powder incorporated into different pellets, their thicknesses will differ, and this fact must be taken into account in the application of Beer's law. Weight of the pellet is proportional to thickness, hence it can be used as a corresponding factor. Also, the concentration of lipoprotein can be expressed as μg/mg NaCl. Beer's law may be written as

$$\text{Absorbance} = A = a \times b \times c$$

where a = absorptivity
b = sample thickness
c = concentration

Substituting expressions for thickness and concentration

$$A = a \times (k.mg) \times (\mu mg/mg) = k' \times \mu g$$

The dimension of either a, the absorptivity, or k', the calibration coefficient, is then $(\mu g)^{-1}$, or absorbance per μg. A plot of the data obtained in this manner is shown in Fig. 8. Although the highest concentration point was not obtained in duplicate, other calibration procedures show similar curvature in the upper concentration range.

A second calibration method is carried out in the manner described, using a commercial oral fat emulsion (Ediol, Schenlabs, New York, N. Y.) instead of chylomicrons as a standard material. When corrected for the molecular weight of the fat (coconut oil) and the estimated fat content of chylomicrons, the slope of the Ediol calibration curve is about 10% higher than that of chylomicrons, and shows less curvature.

In a third calibration procedure, mixtures of triglyceride and KBr are prepared by adding aliquots of a triglyceride solution in a volatile organic solvent (CCl_4 or $CHCl_3$) to a dry bed of KBr powder, mixing as a slurry, and allowing the solvent to evaporate on a 50C

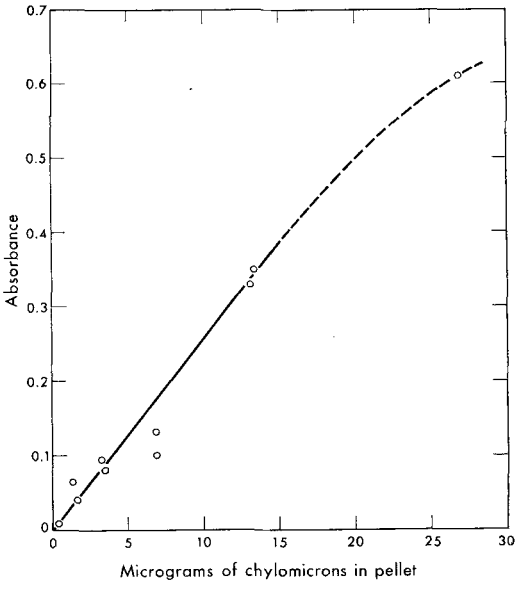


FIG. 8. Calibration curve for infrared analysis of chylomicrons. Amounts of chylomicrons were determined from known dilutions of an analyzed stock preparation of chylomicrons (sample A).

hot plate. Pellets are then pressed from these mixtures, for which concentrations of triglycerides are expressed as μg/mg of KBr. A calibration curve obtained by this method had about the same initial slope as the Ediol curve, but exhibited earlier and more pronounced curvature.

Measurement of the absorbance of the 1742 cm^{-1} peak is made from a recorded curve (scan rate ca. 200 cm^{-1}/min), on which a background is drawn to simulate the shape of the blank (zero triglyceride) curve (see Fig. 7). The peak occurs on the side of a water absorption band, which is somewhat variable in intensity because of nonreproducibility in the freeze-drying process. However there is no difficulty in estimating the contour of this band. A more serious difficulty, which is mainly responsible for the curvature of the calibration curves, is the low order of energy available. In addition to the 70% loss in the beam condenser itself, there are additional losses in the pellet that are subject to considerable variability. Thus the energy available as background may be only 10-20% of normal, and at high absorbances the remaining signal may be as little as 1%. Some compensation is possible by increasing the gain and slowing the scan rate. Unfortunately these compensating measures were not taken in running this initial series of samples, and several of them were too high in chylomicrons to analyze under these conditions.

RESULTS AND DISCUSSION

Table I presents duplicate elemental analyses of chylomicron samples isolated from 16 normal nonfasting males, aged 35–49 years. For clarity, the results are presented in terms of total NCH mass present in 0.1 ml fraction. Correcting this value to serum concentrations involves a 3% increase because of dilution after salt addition and a 12% increase for lipoprotein mass other than the elements N, C and H. Thus, the mean values of our series are 9.62 $\pm$ 11.18 μg NCH/.1 ml fraction or 11.00 $\pm$ 12.78 mg/100 ml total lipoprotein. A standard error of measurement calculated from all duplicate analyses presented in Table I is approximately $\pm$ 0.6 μg/0.1 ml. However, in consideration of the errors in the total procedure and the data from seven repeat analyses, an error of measurement of $\pm$ 1-2 μg/0.1 ml is more realistic. Although it is not recommended to delay chylomicron fractionation any longer than necessary after serum withdrawal, it is encouraging to note reproducibility of results, even after 62 days' serum storage under N_2 at 4C.

TABLE I
Chylomicron Quantitation by Total NCH Elemental Analysis

Sample	Σ NCH, μg/0.1 ml 1	2	Difference, μg/0.1 ml	Fractionation (days after blood withdrawal)
1	15.95	15.91	0.04	8
1 R[a]	19.51	18.78	0.73	15
2	12.67	12.60	0.07	8
3	32.60	33.48	0.88	1
3 R[a]	34.44	33.17	1.27	8
4	0.61	0.72	0.11	1
5	14.03	13.85	0.18	1
6	3.46	2.53	0.93	1
7	0.43	0.97	0.54	8
8	4.46	5.05	0.59	8
9	33.46	32.77	0.69	1
10	0.82	-0.87	1.69	8
11	-0.95	-0.72	0.23	8
12	1.75	1.45	0.30	1
13	0.40	0.23	0.17	1
14	12.97	11.57	1.40	8
15	25.89	25.44	0.45	8
15 (Dupl)[b]	28.17	25.88	2.29	8
15 R 1[a]	26.73	26.27	0.46	62
15 R 2[a]	27.85	28.23	0.38	62
15 R 3[a]	25.85	25.23	0.62	62
15 R 4[a]	25.43	25.37	0.06	62
16	-0.02	-1.18	1.16	8

[a] Reruns.

[b] Duplicate analyses of same fractionation. Standard error of measurement 16 cases $\pm$ 0.54 μg/0.1 ml, all cases $\pm$ 0.61 μg/0.1 ml.

TABLE II

Comparison of Chylomicron Fraction Concentrations by Infrared Spectrometry and NCH Elemental Analysis

Sample No.	Serum concentration, mg/100 ml Infrared	NCH
1	...	18.4
2	...	14.6
3	...	38.1
4	0.4	0.8
5	...	16.1
6	1.1	3.5
7	0.6	0.8
8	5.8	5.5
9	...	38.2
10	0.8	0.0
11	< 0.4	-1.0
12	3.6	1.9
13	3.4	0.4
14	8.9	14.2
15	...	29.6
16	< 0.4	-0.7
Mean values, 10 samples	2.5	2.5
Mean values, 16 samples (NCH data)	11.0 ± 12.8.	

In addition to the mean NCH data for all samples, Table II presents the infrared data for ten serum samples. For comparison, the mean value of the NCH data corrected to total lipoprotein concentration is given. Only the infrared values below 10 mg/100 ml are included and the highest of these are in the upper range of the calibration curve, where accuracy is beginning to deteriorate. More recent work indicates that improvement is possible in both sensitivity and reproducibility when more appropriate instrumental conditions are used.

A principal reason for using an overlayering technique in preparing the salt gradient was to permit chylomicron isolation in a single run, without the difficulties of washing (8), to eliminate contamination and to minimize artifacts (20). In a gradient prepared by successive layering at the bottom of the tube with a spinal needle, the last operation consists of layering the sample into the bottom region. It is almost impossible in this step to avoid contamination of all layers of the gradient including the uppermost ones as the needle is lowered and raised. Results given in Fig. 9 show the freedom from contamination obtained by our procedure. Values are given for successive fractions from a 3½ hr static control at 1 × g. The essential absence of contamination in the first 3 ml fractions in spite of a very large chylomicron concentration indicate that during our fractionation, little or no material is transferred from the bottom serum layer to the top fraction by convection or diffusion. Further, of the sixteen chylomicron samples, six top fractions that were essentially water-clear showed duplicate total NCH values of less than 1 μg/0.1 ml, suggesting no contamination above this magnitude. With the exception of case 13, these very low lipoprotein concentrations were confirmed by the infrared analysis.

One of the occasional difficulties was a contamination of 5-8 μg which may occur during manipulation. However, such a contamination may be present in the folds of the Coleman type boats, presumably resulting from their cardboard packaging. Although all boats were washed 3 times with redistilled hexane in dustproof containers, this potential contamination was not eliminated. When such contamination was observed in any low level sample, a new pair of duplicate samples were run the following day.

A potentially valuable analytical result from NCH elemental analysis of lipoproteins is a precise evaluation of protein content. If the N/NCH ratios for the lipid and protein moieties of a given lipoprotein class are known, an experimental value of N/NCH for the total lipoprotein uniquely determines the protein content. Table III presents elemental analyses of the chylomicron fraction from sample A at mass levels of from 60–500 μg. For the purposes of accurate protein evaluation, a sample mass of from 250–700 μg is needed. The N/NCH ratio for the lipid moiety (0.0010) was based upon a lipid analysis of the chylomicron fraction in which the 4.9% phospholipid content was all assumed to be lecithin. The corresponding ratio of 0.2000 for protein was calculated from the amino acid composition of S_f 0–20 lipoprotein (21) cor-

	Sample ml		Concentration ΣNCH (μg/0.1 ml)	
	1	2	1	2
Analysis I	0.1008	0.0991	220.01	216.09
Analysis II	0.0996	0.0984	205.24	206.50

CHYLOMICRON, SAMPLE B, Static contamination, 3½ hours

Milliliters	ρ_{20} (g/ml)	Sample ml 1	Sample ml 2	Concentration ΣNCH (μg/0.1 ml) 1	Concentration ΣNCH (μg/0.1 ml) 2
0					
	1.0095	0.0937	0.0992	-0.04	0.22
1					
	1.0143	0.0929	0.0964	1.15	0.39
2					
	1.0210	0.0925	0.0926	0.82	2.67
3					
	1.0303	0.0986	0.0969	11.88	10.99
4					
	1.0392	0.0898	0.0961	91.70	81.83
	1.0464	0.0450	0.0453	1766	1743
5					
	~1.076	0.0208	0.0204	6272	6362
6					

FIG. 9. Static contamination of chylomicron fraction from serum sample B (high in chylomicron content).

TABLE III

Protein Content[a], Chylomicron Sample A

Chylomicron concentration	Sample volume, ml	Σ NCH (μgm)	Σ N (μgm)	N/NCH	% P
1.0 c_o	0.01314	372.21	1.72	0.0046	2.11
1.0 c_o	0.01342	366.75	1.78	0.0048	2.23
1.0 c_o	0.01471	432.46	2.15	0.0050	2.35
1.0 c_o	0.01496	424.36	2.08	0.0049	2.29
1.0 c_o	0.01727	482.78	2.27	0.0047	2.17
1.0 c_o	0.01785	481.78	2.59	0.0054	2.58
0.1 c_o	0.10089	272.54	1.21	0.0045	2.05
0.1 c_o	0.10075	272.61	1.33	0.0049	2.29
0.05 c_o[b]	0.10051	123.68	0.79	0.0064	3.17
0.05 c_o[b]	0.09306	112.00	0.86	0.0077	3.92
0.025 c_o[b]	0.10221	60.52	0.46	0.0075	3.80
0.025 c_o[b]	0.10304	59.63	0.39	0.0065	3.22

Mean value ± Standard Deviation (8 samples) 2.26 ± 0.21 % protein.

[a]Calculated N/NCH chylomicron lipid = 0.0010, Sf 0-20 protein 0.2000.

[b] Values below 200 μg Σ NCH are not included in calculations.

rected for H_2O removal from a 100,000 mol wt peptide chain. For the 8 analyses within this range, N/NCH ratios of from 0.0045–0.0054 corresponding to 2.05–2.58% protein, respectively, were obtained, in agreement with composition data reported by others (3,5,8,12). Such an evaluation of protein content with a relative error of the order of 0.2% compares favorably with such techniques as the Lowry (22) microdetermination of protein, with the advantage of simplicity of procedure.

We have experienced no difficulty in the analysis of serum containing high concentrations of chylomicrons with the NCH method. For example, neither serum dilution nor modification of the isolation procedure was required in the analysis of samples A (3100 mg/100 ml) and B (220 mg/100 ml). However, the infrared assay cannot be used without dilution for serum chylomicron concentrations exceeding 10 mg/100 ml. The higher sensitivity of the infrared analysis permits analysis of very low chylomicron levels, including the potential measurement of chylomicrons from fasting sera. On the other hand, elemental analysis allows evaluation of total NCH mass for any chylomicron concentration above 10 mg/100 ml. Because of the enormous range of chylomicron concentrations that may occur in man and experimental animals, both methods of mass assay may be required, one complementing the other.

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SHORT COMMUNICATIONS

Contamination of Methyl Ester Preparations During Methylation of Fatty Acids on an Ion Exchange Resin

ESTERIFICATION OF FATTY ACIDS by the method of Hornstein et al. (1) appears to offer a clean preparation of methyl esters. In this procedure, the fatty acids released upon saponification are selectively adsorbed on the sodium hydroxide pretreated anion exchange resin Amberlite IRA 400. Then the acids are methylated on the resin with anhydrous methyl alcohol-hydrochloric acid mixture. Impurities in the hydroxyl form of the resin are removed by washing in succession with water, ethanol, and petroleum ether before adsorption of the fatty acids. After adsorption of the fatty acids, products of saponification other than fatty acids are removed from the resin by washing three times with petroleum ether. However, in this laboratory it was observed that when various lots of this resin were employed some methyl ester preparations gave a distinct peak between undecanoate and dodecanoate on gas-liquid chromatograms obtained with a diethylene glycol adipate column at 200C. Seemingly this peak did not belong to the fatty acid family.

1) Reference methyl palmitate (Applied Science Labs) dissolved in 75 ml of petroleum ether and stirred 15 min with 10 g of resin which had been converted to the hydroxyl form and washed according to Horstein et al. (1). The procedure included washing three times with 25 ml of petroleum ether. The methyl ester solution in petroleum ether was concentrated to 0.5 ml before GLC.

2) Same as 1 except the petroleum ether did not contain methyl esters.

3) Same as 2 except the resin was washed once more with 75 ml of petroleum ether.

4) Same as 3 except the resin was washed 2 more times with petroleum ether.

5) Reference methyl palmitate dissolved in petroleum ether and stirred with resin treated as described in (4).

6) Reference methyl palmitate dissolved in petroleum ether and concentrated to 0.5 ml before GLC.

Experiments illustrating the source of this contaminant and how to remove it are given in Fig. 1. As can be seen in chromatograms 1 and 2, the contaminant was present in the hydroxyl form of the resin. This resin had been washed with water, ethanol, and petroleum ether as described by Hornstein et al. (1). However, as shown in chromatograms 3, 4, and 5 of Fig. 1, three additional 15-minute washings with 7.5 volumes of petroleum ether were necessary in order to remove the contaminant.

Control experiments showed that the contaminant was not present in any of the other reagents used. Neither was the contaminant removed by washing the chloride form of the ion exchange resin with petroleum ether prior to conversion of the resin to the hydroxyl form.

Evidently the conditions described by Hornstein et al. (1) are not adequate to remove the contaminant. More thorough washing with petroleum ether is required. The conditions described in this communication offer a procedure for the effective removal of this interfering material.

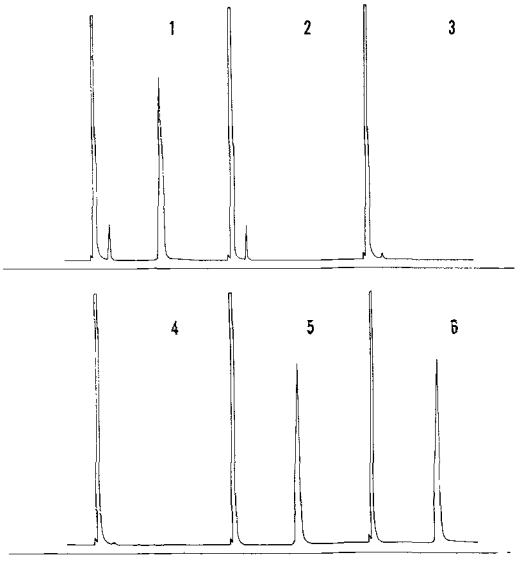

FIG. 1. GLC illustration of the presence of a contaminant in the hydroxyl form of Amberlite IRA-400 ion exchange resin.

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Lipid Droplets and Glyceryl Ether Diesters in Ehrlich Ascites Cells Grown in Tissue Culture

A SIGNIFICANT QUANTITY of an unidentified lipid was recently found in Ehrlich ascites cells (EAC) and other tumors by Snyder and co-workers (1). This class of lipids has now been identified by Wood and Snyder (2) as glyceryl alkyl ether diesters; only the 1-isomeric form of glyceryl ethers was obtained after deacylation of the lipid by saponification and $LiAlH_4$ reduction. The identification was based on thin-layer chromatography, gas-liquid chromatography, infrared spectroscopy, and nuclear magnetic resonance of the intact and deacylated glyceryl ether. Such nonphospholipid ethers can not be seen in normal tissues from healthy or tumor-bearing animals under similar chromatographic loads (1). This note describes experiments designed to determine whether glyceryl ether diesters can be synthesized by EAC grown for extended periods and after multiple transfers in tissue culture media containing no trace of alkoxyglycerols.

The cells were grown in screw-cap serum bottles (Kimax-14250) containing 15-16 ml culture medium 199 (Microbiological Associates, Inc.) with calf serum (10%), K salt of penicillin G (330 units/ml), and streptomycin sulfate (100 μg/ml). The medium was adjusted to a pH of 7.3 ± 0.1. All solutions, glassware, and instruments were sterilized before use, and all procedures were carried out aseptically. The cells initially transferred to culture bottles were collected from our colony of Swiss albino mice (HA/ICR strain) bearing Ehrlich ascites cells. The EAC were grown in

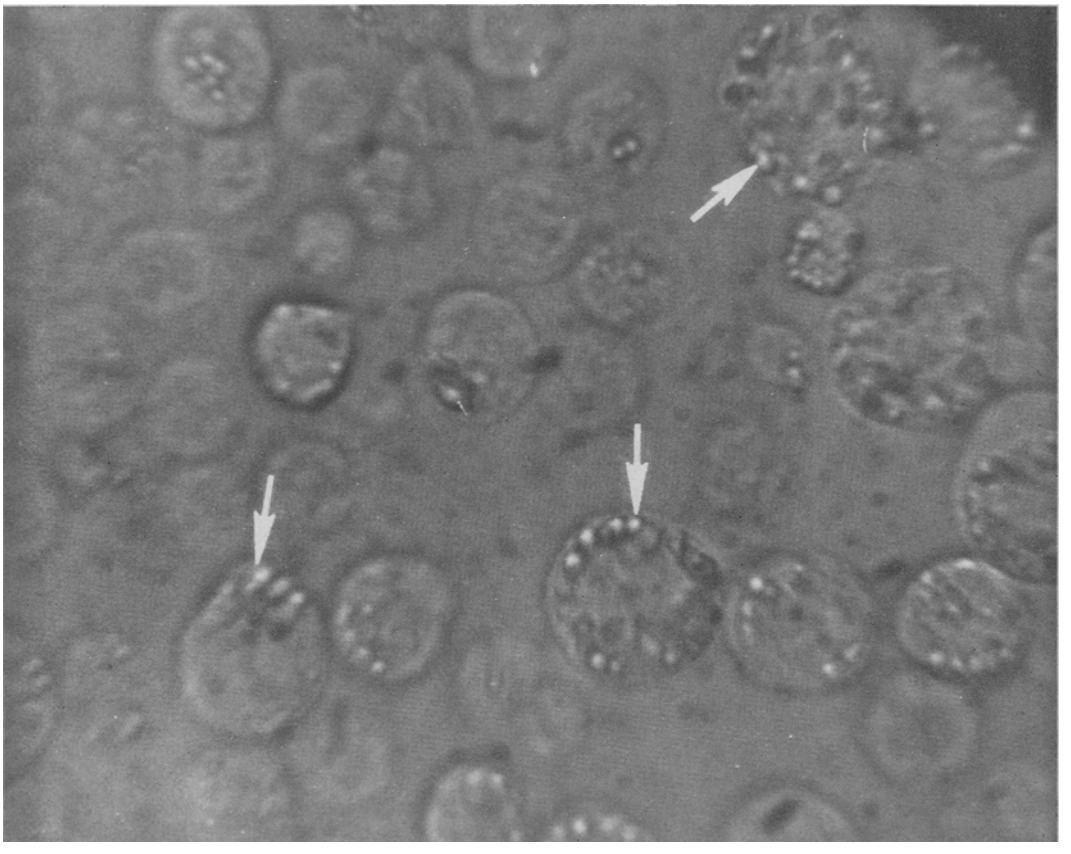

FIG. 1. Ehrlich ascites carcinoma cells after incubating for four days in medium 199 containing 10% calf serum. The lipid droplets show up as light circles within the cells and are stainable with oil red O.

an air atmosphere in an incubator at 37 ± 2C. The culture medium was changed every 3 days and the cell population in each bottle was divided every 6 days. After various periods of growth in the culture bottles, the cells were harvested by centrifugation and washed twice with water. Lipids were extracted from the EAC and their growth media, and thin-layer chromatograms of the total lipids were prepared (1). Occasionally smears of the EAC were stained with oil red O for microscopic examination. In a separate series of experiments the lipids of strain L fibroblasts, originally derived from mouse skin, were grown under identical conditions and examined for the presence of glyceryl ether diesters.

The EAC grown in tissue culture appeared morphologically identical to those grown in the peritoneal cavities of mice. An important observation was the appearance of intracellular lipid droplets stainable with oil red O (Fig. 1) and extractable with diethyl ether. The number of these droplets multiply with increasing duration of incubation. These droplets formed in vitro are indistinguishable from those which have been previously reported in tumor cells grown in vivo (3,4).

Thin-layer chromatography of the total lipids extracted from EAC grown in vitro demonstrated that glyceryl ether diesters occurred in the population of cells even after 4-6 transfers; the TLC patterns were identical to those reported previously for EAC grown in vivo (1). There did not appear to be any relation between the concentration of glyceryl ether diesters and the number of lipid droplets in EAC. The glyceryl ether component was never observed in the lipids extracted from the media either before or after proliferation of the EAC. Furthermore, the glyceryl ether diesters were not found in the L-cells or their growth media under conditions similar to those used for the EAC tissue cultures.

These data rule out the possibility that tumor cells incorporate and concentrate glyceryl alkyl monoethers from other tissues or from dietary intake. The data show clearly that the EAC are capable of synthesizing the lipid ether bond in the absense of other tissues.

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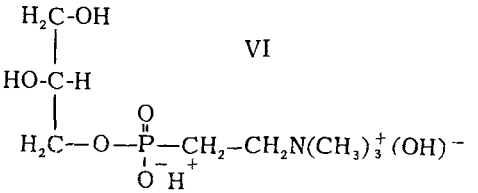
4. Apffel, C. A., and J. R. Baker, Cancer *17*, 176 (1964).

[Received Dec. 14, 1966]

Phosphonolipids. IX. Phosphonolipid Metabolites: Synthesis of L-α-Glyceryl-(2-trimethylammoniumethyl) Phosphonate

IN THE FOLLOWING we report briefly a chemical synthesis of a *phosphonic acid analogue* of L-α-glyceryl-phosphorylcholine, *viz.* L-α-glyceryl - (2-trimethylammoniumethyl) phosphonate (Formula 1). We believe that this compound possesses considerable interest as a possible intermediate in phosphonolipid metabolism, as saponification product for the identification of naturally occurring phosphonolipids, and as starting material for the chemical and enzymatic synthesis of phosphonic acid analogues of saturated and unsaturated L-α-lecithins.



Formula 1. L-α-Glyceryl-(2-trimethylammoniumethyl)phosphonate

The L-α-glyceryl-(2-trimethylammoniumethyl)phosphonate (VI) was obtained by condensing D-acetone glycerol (I) with bromoethylmetaphosphonate (II, Calculated: P 18.12, Br 46.75. Found: P 18.08, Br 46.41) in boiling benzene, removing the acetone group of acetone L-α-glyceryl-(2-bromoethyl)phos-

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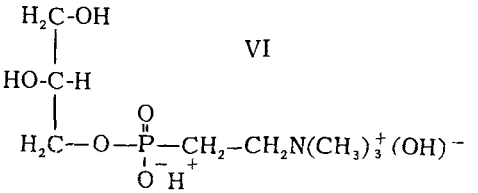

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phonate (III) by acid hydrolysis (pH 1-2), treating the L-α-glyceryl-(2-bromoethyl)phosphonate (IV) in dimethylformamide with trimethylamine at 60-65C for 3 days, and isolating the L-α-glyceryl-(2-trimethylammoniumethyl)phosphonate as cadmium chloride complex (V). The preparation of compound V was carried out without isolating compounds II, III or IV in a pure state. The over-all yield of compound V was 37.5% *of theory* (calculated for acetone glycerol). *Anal.* Calcd. for $[C_8H_{22}O_6NP]_2[CdCl_2]_3$ (1068.5): C 17.98, H 4.15, N 2.62, P 5.80, Cd 31.56. Found: C 16.92, H 4.11, N 2.63, P 5.74, Cd 31.49, $[\alpha]_D$ –0.6C in water (c 7). Vincinal-Glycol Titration: An aqueous solution of compound V was freed of cadmium chloride with potassium carbonate, and the amount of L-α-glyceryl-(2-trimethylammoniumethyl)phosphonate in the filtrate was ascertained by a phosphorus determination. An aliquot of the solution containing 0.0355 mmole of the monoester consumed 0.0350 mmole (98.5%) of periodic acid.

Removal of the cadmium chloride moiety of compound V by treatment of its aqueous solution with a 1:1 mixture of Amberlites IR 45 and IRC 50 (H form) gave L-α-glyceryl-(2-trimethylammoniumethyl)phosphonate (VI) in a yield of 80%. Over-all yield 30% based on acetone glycerol. The highly hygroscopic, glass-like material is soluble in water, methanol and 99% ethanol, but insoluble in chloroform, acetone, ether or benzene. $[\alpha]_D^{24}$ –1.5C in water (c 3.6). A 0.1 molar solution of compound VI in carbon dioxide-free water has a pH of 4.3 at 24C. Anal. Calcd. for $C_8H_{22}O_6NP$ (259.2): C 37.06, H 8.56, N 5.40, P 11.95. Found: C 37.13, H 8.61, N 5.42, P 11.99. Vicinal-Glycol Titration: 0.240 mmole of compound VI consumed 0.237 mmole (98.7%) of periodic acid.

ACKNOWLEDGMENT

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[Received Nov. 28, 1966]

Effect of Phenazine Methosulfate on the Incorporation of C-Labeled Glucose into Lipids of Newborn Brain

IN THE NEWBORN dog cerebral cortex, neurophysiologic function is minimal, possibly due to the incomplete formation of lipid-containing neuronal substructure such as myelin and nerve endings. In mature cortex, glucose, the primary energy source, is metabolized principally via glycolysis and the energy derived supports function. An alternate route for glucose metabolism, the hexose monophosphate pathway (HMP), has previously been shown by McLean to be intimately associated with lipid biosynthesis in lactating mammary gland, where stimulation of glucose incorporation into lipids was paralleled by increased HMP activity (1). In this communication, data are presented to demonstrate that phenazine methosulfate, an artificial electron acceptor which stimulates HMP activity in newborn brain (2), inhibits the incorporation of ^{14}C-glucose carbon into brain lipids.

Dogs 1-3 days old were decapitated and slices of cerebral cortex (gray matter) were prepared and incubated at 37C in standard Warburg vessels (see Table I for conditions). After 100 min. incubations, the reactions were stopped by quick-freezing at –75C (Cellosolve-dry ice mixture). The frozen tissues were homogenized, extracted with chloroform/methanol (2/1), and the nonlipid contaminants removed on Sephadex G-25 (3). The individual phospholipids were determined after separation by two-dimensional thin-layer chromatography (4).

In Table I are recorded the relative amounts of ^{14}C-glucose carbon incorporated into newborn brain lipids. Maximum activity occurred in the phospholipid fraction, while free fatty acids and cholesterol ester contained the lowest amount of isotope. No attempt was made to distinguish between net lipid synthesis and turnover because of the variation in individual lipid pools. With phenazine methosulfate a 4- 5-fold decrease was observed in the ^{14}C-incorporation into neutral lipids, whereas the

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TABLE I

Incorporation of ^{14}C-Glucose [U.L.] into Newborn Brain Lipids[a]

	Control[b] (μc/dry gram)	Phenazine Methosulfate[c] (μc/dry gram)
Phospholipid	3.21	1.62
Triglyceride	1.30	0.30
Cholesterol	0.91	0.16
Cholesterol ester	0.55	0.10
Free fatty acids	0.20	0.05

[a]Results are expressed as averages of three experiments.

[b]Counting yield (%) = $\frac{\mu c \text{ in total lipids/300 mg fresh brain}}{\mu C \ ^{14}C\text{-glucose added/flask}} \times 100$

Counting yields: Control, 1.98%; phenazine methosulfate, 0.72%.

[c]Phenazine methosulfate concentration: [1 x 10-4M].

The incubation mixture (200-300 mg fresh weight of brain in 2.0 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4) contained 10 μc of ^{14}C-glucose [U.L.]; substrate 5 mM cold glucose. Gas phase was 95% oxygen-5% carbon dioxide. The lipids were chromatographed by one-dimensional TLC in 4/1 chloroform/methanol with 1% acetic acid on plate spread with silicic acid-magnesium silicate (9/1). Authentic standards were used for identifications. Developed plates were exposed to iodine vapors and the silicic acid phospholipids suctioned into 20 ml scintillation vials. The silicic acid-lipid mixtures were then counted in a liquid scintillation spectrometer.

TABLE II

Incorporation of ^{14}C-Glucose [U.L.] Into Newborn Brain Phospholipids

	Control			Phenazine Methosulfate		
	μmole P dry g	mμC dry g	S.A.[a]	μmole P dry g	mμC dry g	S.A.
Phosphatidic acid	2.2	57.3	26.0	2.4	62.9	26.2
Phosphatidyl serine	26.1	80.2	3.1	23.8	56.7	2.4
Phosphatidyl choline	124.6	917.1	7.4	118.1	227.8	1.9
Phosphatidyl Ethanolamine	56.7	327.9	5.8	53.3	97.1	1.8
Phosphatidyl inositol + sphingomyelin	25.0	150.0	6.0	23.6	63.9	2.7
	234.6			221.2		
Total sample	265.0			280.5		
% Recovery	88.6%			78.8%		

[a] Specific activities are expressed as mμC/μmole phosphorus. Phospholipids were separated using chloroform/methanol/30% aqueous ammonia 65/35/5 followed by chloroform/acetone/methanol/acetic acid/water 5/2/1/1/0.5 and the phosphorus of each spot determined (4). The lipid samples for radioisotope assay were detected on duplicate plates with iodine vapor and the silicic acid-lipid mixtures collected and counted as noted in the legend for Table I.

phospholipid counts were reduced by one half.

Phosphatidic acid had the highest specific activity among the phospholipids (Table II) but was of relatively small pool size. Phosphatidyl choline and phosphatidyl ethanolamine were the largest constituents in this fraction based upon phosphorus analysis. Although comprising the bulk of the total radioisotope content, these phospholipids have specific activities only 20-30% of phosphatidic acid in control tissue. In the presence of phenazine methosulfate, there occurred a 5-fold decrease in isotope content in the major phospholipid components, a difference not observed in their tissue concentrations. Since such change resulted in a fall in specific activity, we interpret this to indicate a phenazine methosulfate inhibition of turnover rather than new synthesis.

Phenazine methosulfate stimulates the direct oxidation of glucose in newborn brain but inhibits the incorporation of ^{14}C-glucose carbon into brain lipids. The observation that phosphatidic acid maintains a relatively constant and high specific activity, even when phospholipid turnover is generally decreased, suggests that either phosphatidic acid formation precedes the site affected by phenazine methosulfate or that compartmentation renders it inaccessible. Current studies in this laboratory also indicate concomitant decreases in the incorporation of ^{14}C-glucose carbon into brain nucleic acid and protein with phenazine methosulfate. Therefore, the effects reported here may not be specific for inhibition of lipid metabolism but reflect a general action of phenazine methosulfate on processes requiring an adequate energy supply.

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Elimination of Contaminants During the Isolation of Fatty Acid Esters by Preparative Gas-Liquid Chromatography

THE ISOLATION of pure fatty acids by preparative gas-liquid chromatography (GLC) is complicated by the persistence of traces of the preceding acids even though the chromatogram may show clear separations. This becomes intolerable in radioactive tracer studies in which the contaminating acid may have a relatively high degree of radioactivity. Elovson (1) claims to have circumvented this difficulty by rechromatography. In our hands this does not completely remove all the contaminating materials.

Reiss et al. (2) have reported a collection system by which they isolate "a relatively pure sample." Actually, the chromatogram of the octanoic acid they isolated shows clearly the presence of heptanoic acid.

The difficulty in the isolation of pure fatty acids by GLC is most probably due to traces of the preceding emerging acids which remain in the outlet tubes. This difficulty can be circumvented by injecting from 10-15 μl of hexane at such a time that the peak due to hexane appears just before the peak of the acid to be collected.

It is the usual procedure for fatty acid isolation in our laboratory to first isolate groups of acids of common carbon numbers by GLC on a nonpolar liquid phase, and to subsequently separate them into acids of differing degrees of unsaturation by silver-ion thin-layer chromatography. As is the case with individual fatty acids, the groups of common carbon number acids are usually contaminated with the preceding group.

The following example demonstrates the efficacy of the technique. Methyl esters of rat endogenous fatty acids of adipose tissue triglycerides were separated and collected by the procedure described by Wood and Reiser (3). (Aerograph A-90-P Gas Chromatograph. Column: 12 ft × 3/8 in. coiled copper tubing packed with 25% SE-30 on Chromosorb W, 50-100 mesh; temperature 220C; helium flow rate 60 ml/min.) Fig. 1A represents the analysis of the 18 carbon acid ester fraction by an analytical gas chromatograph (Research Specialties Model 600, Warner-Chilcott Laboratories Div., Richmond, Calif.) equipped with a flame ionization detector. The presence of palmitate and palmitoleate can be seen. However, when the preparative equipment was injected with 10 μl of hexane after the emergence of the C_{16} group but just before the C_{18} fraction, the analytical chromatogram (Fig. 1B) of the latter contained no peaks representative of C_{16} acids.

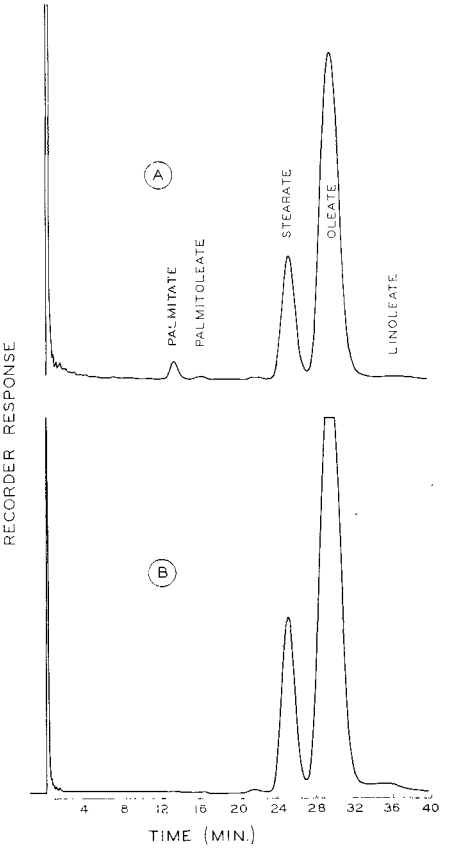

FIG. 1. Gas-chromatographic analysis of the fatty acid methyl esters collected on preparative gas chromatography: *A*, without hexane injection; *B*, after injecting 10 μl of hexane. Column: 6 × 1/4 in. copper packed with 15% diethylene glycol succinate polyester on Gaschom RZ, 60-80 mesh (Applied Science Labs, State College, Pa.). Column temperature 180C; helium flow 50 ml/min.

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Erratum

"Detection of Phthalate Esters as Contaminants of Lipid Extracts from Soil Samples Stored in Standard Soil Bags," *Lipids,* January, 1967. On page 86, Figure 1 should appear as corrected at the right.

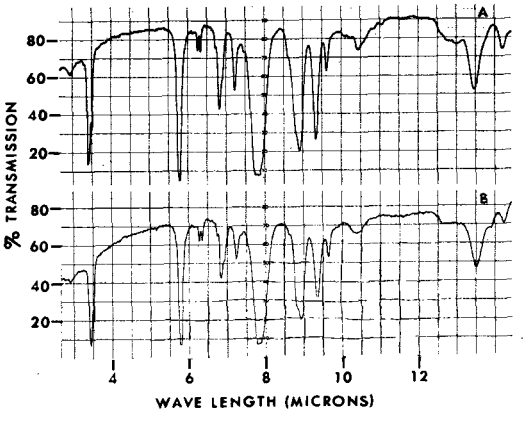

FIG. 1. *A,* infrared spectrum (1.82% in potassium bromide) of the phthalate ester isolated from a soil sample; *B,* authentic didecylphthalate.

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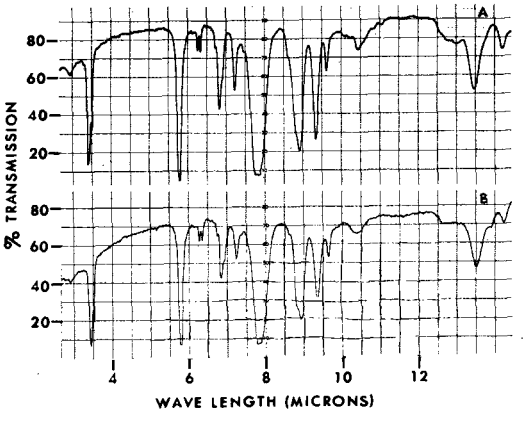

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GLC and TLC Analysis of Isopropylidene Derivatives of Isomeric Polyhydroxy Acids Derived from Positional and Geometrical Isomers of Unsaturated Fatty Acids

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ABSTRACT

Gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) were used to investigate the isomeric positional geometrical isopropylidene derivatives of nine isomeric dihydroxy esters, four isomeric methyl 9,10-12-trihydroxystearates, and eight isomeric methyl 9,10-12,13-tetrahydroxystearates prepared from unsaturated fatty acids. The isopropylidenes derived from *cis* and *trans* monounsaturated fatty acids were easily separated on both polar and nonpolar columns. Positional isopropylidenes derived from positional isomers of monounsaturated fatty acids were not separated on either liquid phase but were resolved by TLC.

Four of the eight isomeric isopropylidenes derived from the four geometrical isomers of linoleic acid were resolved on the polar column; the other four isomers eluted as a single peak. The four isomeric isopropylidene-trifluoroacetate derivatives derived from ricinoleic and ricinelaidic acids were also resolved on the polar column. GLC analyses were carried out with liquid phases of ethylene glycol succinate methyl silicone polymer (EGSS-X) and methyl silicone polymer (SE-30) packed columns. Isopropylidenes, in addition to their applicability for the resolution of polyhydroxy acid mixtures, are particularly useful for the determination of double bond positions and geometrical configurations of fatty acids without cleavage.

INTRODUCTION

THE QUANTITATIVE DETERMINATION of double bond position and geometric configuration of unsaturated fatty acids in a mixture is one of the most difficult problems for the lipid chemist. Such a determination now requires the isolation of each acid, followed by determination of configuration by infrared spectroscopy (1), gas-liquid chromatography (GLC) (2), or argentation thin-layer chromatography (TLC) (3), and subsequent analysis of double bond position by degradation and product analysis. The fragments of degradation formed by periodate-permanganate oxidation (4) or ozonolysis (5,6,7) are analyzed by GLC. Attempts to determine the double bond position of intact fatty acids or derivatives have been only partially successful: quantitative resolution of suitable monoene mixtures by argentation TLC has been reported (8), and the positional isomers of methyl octadecanoate beyond the $\Delta 9$ position have been resolved by capillary GLC (9).

Isopropylidene derivatives of 1-monoglycerides (10) and 1-glyceryl ethers (11) have been used for their GLC analysis. Recently isopropylidene derivatives derived from unsaturated esters (12) and hydrocarbons (13) have been used in an attempt to determine the double bond position by tandem GLC-mass spectrometry. Only the details of the mass spectrometry data have been published, and they appear to be of little value, if any, in the quantitative identification of a mixture of positional isomers. In view of the unexplored potential of the isopropylidene derivatives for determining fatty acid double bond position and configuration by GLC and TLC, experiments to determine such applicability were carried out.

EXPERIMENTAL

Materials

The palmitoleic, oleic, elaidic, petroselenic, *cis*- and *trans*-vaccenic, 11-eicosenoic, erucic, linoleic, linoelaidic, ricinoleic, and ricinelaidic acids of high purity were obtained from the Hormel Institute, Austin, Minn. The *cis,trans*- and *trans,cis*-linoleic acids were gifts from O. S. Privett. The system of nomenclature and the physical properties of the polyhydroxy acids used in this study have been previously described (14). Glass-distilled solvents were obtained from Burdick and Jackson Laboratory Inc. Other reagents and materials were

[1] Under contract with the U. S. Atomic Energy Commission.
[2] An operating unit of Oak Ridge Associated Universities Inc.

reagent grade and used without further purification.

Gas-Liquid Chromatography

Analyses were made with an Aerograph Model 204 Gas Chromatograph. Pyrex columns (5 ft x ⅛ in.) packed with 15% of ethylene glycol succinate methyl silicone polymer (EGSS-X), coated on 100-120 mesh Gas-Chrom P, and a stainless steel column (5 ft x ⅛ in.) packed with 5% methyl silicone polymer (SE-30), coated on 60-80 mesh Chromosorb W, were used. The polar EGSS-X columns were operated isothermally at 180 and 200C for the analysis of the isopropylidene derivatives derived from mono- and di-unsaturated acids. Isopropylidene TFA derivatives derived from ricinoleate and ricinelaidate were analyzed on EGSS-X columns at 185C. The temperature for SE-30 columns was manually programmed from 200-230C for all analyses. Injector and detector temperatures were maintained at 230 and 240C. Air and hydrogen flow rates were regulated to give maximum sensitivity of detectors at 60 cc/min of He carrier gas.

Thin-Layer Chromatography

Uniform 0.5-mm and 0.25-mm layers of Silica Gel G were spread on 20- x 20-cm glass plates with a Colab applicator modified in this laboratory (15). The chromatoplates, after air-drying for 30 min, were activated for 15 min at 110C. The isomeric isopropylidene derivatives derived from ricinoleic and ricinelaidic acid (one free hydroxyl group) were chromatographed in chloroform. Hexanediethyl ether-30% aqueous ammonium hydroxide 90:12:0.25 (v/v/v) was used for chromatography of the other samples. All chromatography was carried out in saturated chambers. Separations on preparative plates were visualized by spraying with 0.2%, 2',7'-dichlorofluorescein in ethanol and viewing under UV light. Analytical plates were charred according to the procedure of Privett and Blank (16), and results were documented by photography.

Derivative Preparation

Potassium permanganate hydroxylations were carried out as described previously (14). The hydroxy acids were then methylated with an ethereal solution of diazomethane, prepared according to De Boer and Backer (17). Isopropylidene derivatives were prepared by a modified procedure of Hanahan et al. (11). One to 10 mg of the hydroxy esters were placed in a screw-cap vial with 1 ml of acetone and 1 μl of 60% perchloric acid. After 15 min the perchloric acid was neutralized with excess ammonium hydroxide. The total mixture was used directly for GLC analysis, with the exception of the isopropylidene derivatives of the trihydroxy esters. These samples were evaporated to dryness, and the free hydroxyl group was trifluoroacetylated (18) and analyzed by GLC.

RESULTS AND DISCUSSION

Hydroxylation

Quantitative oxidation of the double bond to the diol, illustrated in Step 1 of the reaction scheme, is the most difficult step. The stereospecific oxidation, previously shown to oxidize mixtures of oleic and elaidic acids quantitatively to isomeric diols (14), is incomplete

TABLE I

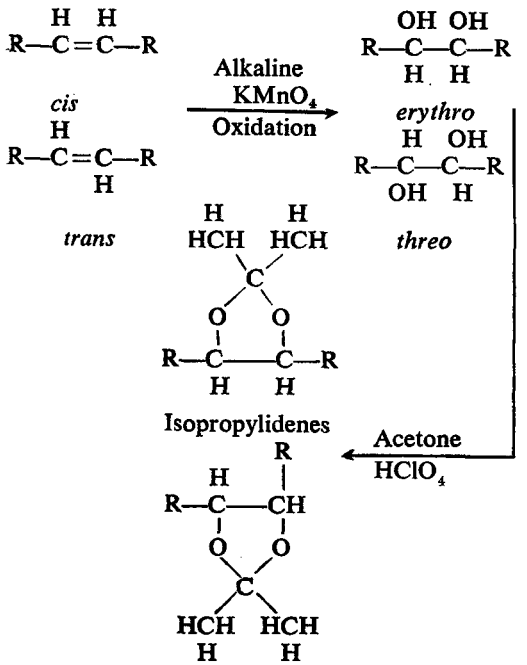
Analysis of Monounsaturated Standard Mixtures of Fatty Acids by GLC Analyses of the Isopropylidene Derivatives Obtained from the Isomeric Dihydroxy Acids, Resulting from Alkaline Permanganate Oxidation [a]

Parent acid	Known weight %	% Found on EGSS-X column [b]	% Found on SE-30 column [b]
		Mixture No. 1	
Palmitoleic	23.5	24.5	24.7
Oleic	22.9	26.1	24.9
Elaidic	21.6	17.8	16.7
11-Eicosenoic	16.7	19.1	20.0
Erucic	15.4	12.4	13.6
		Mixture No. 2	
Palmitoleic	32.9	34.3	35.5
Oleic	26.8	26.2	26.8
Elaidic	18.3	14.7	14.8
11-Eicosenoic	23.0	24.8	22.9

[a] Comparison of known weight percentages of the parent acids with experimental values represents an over-all evaluation of the method. The sum of the possible errors associated with hydroxylation, methylation, derivative preparation, and analysis are incorporated into the experimental values.

[b] Each experimental value represents the mean of three determinations. Percentages were calculated from peak areas obtained by triangulation. The detector response of the isopropylidenes was found to be linear over the concentrations used. The precision and accuracy of the chromatographic data, including the peak area measurements, is estimated to be in the order of ± 2%.

when monoene mixtures of a wider molecular weight range are oxidized (Table I). Incomplete oxidation of the less soluble, longer chain monoenes was encountered. Prolonged reaction time increased the concentration of presumably ketols (monoketo-monohydroxy). Alkaline permanganate oxidation of the geometrical isomers of linoleic acid produced tetraols with different degrees of water solubility. Lyophilization of the neutralized reaction mixture failed to yield tetraols, presumably because oxidation continued during this process. Despite diene hydroxylation difficulties, sufficient quantities of isomeric tetraols of known configuration (14) were obtained for the evaluation of isomeric isopropylidene GLC and TLC separation patterns. Quantitative hydroxylation of mono- and di-enes might be achieved by the osmium tetraoxide procedure referred to by McCloskey and McClelland (12).



Isopropylidene Derivatives

The preparation of the isopropylidene derivatives from the hydroxy esters, Step 2 of the reaction scheme was virtually complete after 5 min of reaction time as determined by TLC; but 15 min of reaction time were allowed to assure completion. This is considerably faster than that reported for other methods (12,19). Contrary to previous reports (19,20), differences in reaction rates of the *threo* and *erythro* isomers could not be detected by the method used. Samples removed from the reaction mixture after neutralization with an excess of ammonium hydroxide were injected directly into the chromatograph. Neutralization is necessary because the build-up of perchloric acid on the columns from samples that had not been neutralized caused virtually complete breakdown of derivatives. One major breakdown product was observed for each of the isopropylidenes derived from oleic and linoleic acids (the only two derivatives chromatographed under these conditions). The retention time of the breakdown product corresponded to that of the original unsaturated ester; however no further identification attempt was made.

GLC Analysis of Isopropylidenes Derived from Monenes

Individual monounsaturated fatty acids and mixtures were hydroxylated; methylated, isopropylidene derivatives were prepared and analyzed directly by GLC without any preliminary purification. The total time required to prepare the derivatives and complete the analysis was less than three hours. Fig. 1 shows two typical chromatogram tracings obtained with SE-30 and EGSS-X liquid phases, depicting the analysis of isopropylidenes derived from monounsaturated fatty esters ranging in chain length from C_{16} to C_{22}. Isopropylidene derivatives derived from *cis* and *trans* monoenes of the same chain length were easily separated on both liquid phases (Figure 1, peaks 2 and 3). The derivatives of the *cis* isomers had the longer retention times, as had been previously reported for isopropylidenes

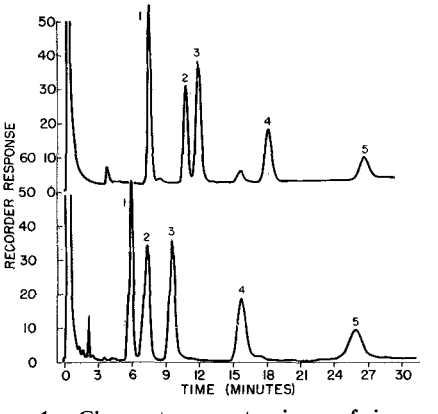


FIG. 1. Chromatogram tracings of isopropylidenes derived from the following monounsaturated fatty acids: (1) palmitoleic; (2) elaidic; (3) oleic; (4) 11-eicosenoic; and (5) erucic. Analyses were made on a 5 ft x ⅛ in. 5% SE-30 column manually temperature-programmed from 200-230C (top) and a 5 ft x ⅛ in. 15% EGSS-X column operating at 187C (bottom).

TABLE II

Relative Retention Times of Isopropylidene-TFA Derivatives and TLC R_f's of Isopropylidene Derivatives, Prepared from Trihydroxystearates Resulting from Akaline Permanganate Oxidation of Ricinoleic and Ricinelaidic Acids

Methyl trihydroxystearate derivative	Relative retention time [a]	TLC R_f [b]
threo-9, 10-*erythro*-10, 12-	1.0 (15 min)	0.5[c]
threo-9, 10-*threo*-10, 12-	1.12	0.4
erythro-9, 10-*threo*-10, 12-	1.25	0.27
erythro-9, 10-*erythro*-10, 12-	1.46[d]	0.43[e]

[a] Analysis made on EGSS-X liquid phase.

[b] One free hydroxyl group.

[c] The R_f of methyl 12-hydroxystearate chromatographed under the same conditions was 0.5 whereas methyl 9, 10-12-trihydroxystearate did not move from the origin.

[d] Contained a second component with a retention time of 1.06.

[e] Showed a double spot.

derived from unsaturated hydrocarbons (13). Mixtures of positional isopropylidene isomers derived from methyl oleate, petroselenate, and *cis*-vaccinate were not resolved on either liquid phase.

Table I shows, for two monoene mixtures, a comparison of known weight percentages with experimental values determined on polar and nonpolar columns. Discrepancies are attributable to incomplete hydroxylation procedure and the lack of standard purity. Presumably a quantitative hydroxylation would make this method applicable for the simultaneous quantitative determination of composition and configuration of a monoene mixture.

Analysis of Isopropylidenes Derived from Monohydroxy-monoenes

A mixture of isopropylidene-TFA derivatives of the four diastereoisomeric trihydroxy esters resulting from the oxidation of ricinoleic and ricinelaidic acids was resolved by GLC on the polar columns. Resolution had not been possible previously with the TFA derivative alone (14). The relative retention times for the isopropylidene-TFA derivatives are shown in Table II. Analysis of the individual isomers revealed a second component (retention time 1.06) in the isopropylidene-TFA derivative of methyl *erythro*-9,10-*erythro*-10,12-trihydroxystearate. The second component was also observed on TLC. Although the unidentified component might be an artifact, other possible explanations include the formation of a 10,12-isopropylidene in addition to the expected 9,10-isomer (not observed in the corresponding *threo*-9,10-*erythro*-10,12-isomer) or the formation of other isomeric forms derived from the original ricinoleic acid.

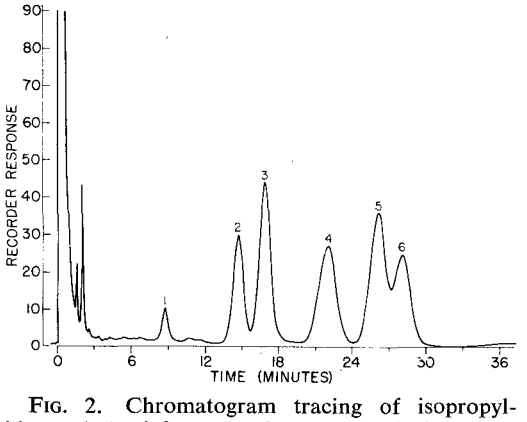


FIG. 2. Chromatogram tracing of isopropylidenes derived from the four geometrical isomers of linoleic acid. The peak numbers are isopropylidenes of (1) oleic; (2 + 3) *cis,cis*-linoleic; (4) *cis,trans*- and *trans,cis*-linoleic; and (5 + 6) *trans,trans*-linoleic acids. Analysis was made on a 5 ft. × ⅛ in. EGSS-X column at 200C.

GLC of Isopropylidenes Derived from Dienes

The four geometrical isomers of linoleic acid, upon oxidation with alkaline permanganate, give rise to eight diastereoisomeric tetrahydroxystearic acids. The GLC analysis of the isopropylidenes prepared from all eight of the tetrahydroxy isomers is shown by the chromatogram tracing in Fig. 2. Preliminary TLC purification was required to remove unoxidized and partially hydroxylated contaminants before isopropylidene preparation. The isopropylidenes derived from the two *threo, threo*-tetrahydroxy isomers (peaks 2 and 3) were resolved and eluted several minutes before the two partially resolved *erythro, erythro* isomers (peaks 5 and 6), and the intermediate forms (the two *erythro, threo* plus the two *threo, erythro* isomers) were eluted as an intermediate single peak (peak 4). The same elution order was obtained on the SE-30 column, but resolution was not so good as that obtained on the EGSS-X liquid phase. The partially resolved isopropylidenes of the two *threo, threo* isomers were completely resolved from the four

threo, erythro-erythro, threo isomers shouldered on the leading side of the single peak containing the two *erythro, erythro* isomers.

The diastereoisomeric pairs of *threo, threo-* and *erythro, erythro*-tetrahydroxystearate TFA derivatives have previously been resolved by GLC (14); however the resolution of a mixture such as that shown in Fig. 2 has not been reported. Such separations make possible the qualitative classification of the original diene double bond configurations into the following isomeric forms: *cis cis, trans trans,* and the indistinguishable *cis trans* and *trans cis* isomers.

TLC of Isopropylidenes

A chromatoplate depicting the resolution of isopropylidene fatty esters derived from monounsaturated positional isomers by TLC is shown in Fig. 3. It may be concluded, on the basis of the number and type of positional isomers examined, that polarity decreases with the increasing distance of the 1,3-dioxolane ring from the carbonyl group. Isopropylidenes derived from *trans* monoenes (lanes 5 and 6) are slightly less polar and migrate ahead of those derived from the corresponding *cis* monoenes (lanes 2 and 3). This order of migration also holds for isopropylidenes derived from dienes with two 1,3-dioxolane rings (lanes 9 and 10). Isopropylidenes derived from *cis,trans* and *trans,cis* dienes had identical R_f's and showed intermediate polarity to those in lanes 9 and 10. The polar materials remaining near the origin (Fig. 3) are products of acetone polymerization that occur upon storage rather than from unreacted hydroxy esters. These solvent products usually do not interfere with analysis; however they can be avoided by extracting the isopropylidenes from the reaction mixture.

The resolution of positional isopropylidene isomers by TLC was unexpected, owing to the lack of resolution by GLC on the polar EGSS-X liquid phase. Other polar liquid phases may prove satisfactory for the GLC resolution of positional isopropylidenes derived from monoenes.

TLC R_f values of isopropylidene derivatives derived from ricinoleic and ricinelaidic acids are shown in Table II. As expected, these compounds, which contain one free hydroxyl group, showed polarities equal to or greater than methyl 12-hydroxystearate. A double spot was observed for the isopropylidene derivative of methyl *erythro*-9,10-*erythro*-10,12-trihydroxystearate, and its possible origin is discussed in the GLC section.

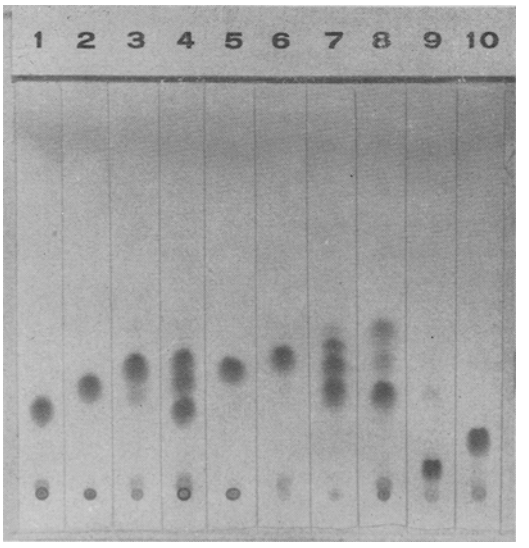


FIG. 3. Thin-layer chromatograms of isopropylidene fatty esters derived from (1) petroselenic; (2) oleic; (3) *cis*-vaccenic; (4) mixture of 1, 2, and 3; (5) elaidic; (6) *trans*-vaccenic; (7) mixture analyzed in Fig. 1; (8) palmitoleic; (9) linoleic; and (10) linoelaidic acids. Solvent system was hexane-diethyl ether-30% aqueous ammonium hydroxide 90:12:0.25 (v/v/v).

Presently GLC and TLC of the isopropylidenes derived from monoenes can be used to determine the homogeneity of the double bond position and configuration of fatty esters without cleavage.

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X-ray Diffraction Study of Some Normal Alkyl Esters of Long-Chain Acids[1]

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ABSTRACT

X-ray powder diffraction data are reported for 15 normal long-chain esters. The compounds represent all combinations of acid and alcohol where the acid portion is n-tetradecanoic, n-hexadecanoic, or n-octadecanoic acid, and the alcohol portion is n-tetradecanol, n-pentadecanol, n-hexadecanol, n-heptadecanol, or n-octadecanol. The individual compounds can be identified and distinguished by the diffraction data. Several of the esters have long spacings that are a linear function of the number of carbon atoms in the molecule and are consistent with a similar function for ethyl esters of long-chain acids. The remainder of the compounds crystallize in other polymorphic forms and therefore do not follow this function.

INTRODUCTION

KOHLHAAS (4) HAS REPORTED the unit cell and space group for hexadecyl hexadecanoate. The methyl and ethyl esters of normal long-chain acids were characterized by powder diffraction by Malkin (6), Francis et al. (2, 3) and Piper et al. (10). However little is known about the crystallography of higher homologs of normal aliphatic esters. This paper reports x-ray diffraction studies for 15 normal long-chain aliphatic esters whose general formula is $CH_3(CH_2)_nCOO(CH_2)_mCH_3$ where n is 12, 14, or 16, and m is 13, 14, 15, 16, or 17. All compounds under study are solid crystalline materials, the melting points of which are above room temperature and therefore suitable for study by x-ray diffraction.

EXPERIMENTAL

Materials and Methods

Sample Preparation. Observed melting-points and literature values (11) for these compounds are included in Table I. The majority of the samples were purchased from Lachat Chemicals Inc. Two samples (octadecyl tetradecanoate and octadecyl octadecanoate) were synthesized at this Laboratory. The commercial samples had to be washed to remove unreacted acids. The odd-chain alcohol esters had to be further purified by passing through a column of silicic acid to remove both unreacted alcohol and acid. Thin-layer chromatography showed no trace of alcohol and acid after this treatment. The samples were then crystallized three

[1] Presented at AOCS Meeting, Philadelphia, October 1966.
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TABLE I
Long Spacings of Normal Alkyl Esters of Long-Chain Acids

Name of compound	Melting point (observed)	Melting point (literature)	Total carbon atoms in molecule	Long spacing Å	No. of orders used
Tetradecyl tetradecanoate	39.1—39.8		28	34.54 ± 0.06	14
Pentadecyl tetradecanoate	45.8—46.4		29	36.70 ± 0.15	4
				35.76 ± 0.05	4
				35.16 ± 0.08[a]	5
				32.67 ± 0.12[b]	3
Tetradecyl hexadecanoate	49.5—49.8		30	36.79 ± 0.01	13
Hexadecyl tetradecanoate	46.4—46.5		30	41.36 ± 0.04	8
Pentadecyl hexadecanoate	55.8—56.4	55.5	31	37.62 ± 0.01	13
Heptadecyl tetradecanoate	50.6—51.4		31	39.83 ± 0.05	8
				37.83 ± 0.06[b]	5
Tetradecyl octadecanoate	51.2—51.8		32	43.44 ± 0.01	14
Hexadecyl hexadecanoate	49.8—50.4	51.6	32	38.90 ± 0.01	15
Octadecyl tetradecanoate	50.5		32	38.98 ± 0.02	16
Pentadecyl octadecanoate	55.4—55.8		33	39.75 ± 0.01	20
Heptadecyl hexadecanoate	57.6—58.2		33	39.84 ± 0.02	18
				36.74 ± 0.08	5
Hexadecyl octadecanoate	57.0—57.6		34	41.34 ± 0.02	15
Octadecyl hexadecanoate	58.3—58.8	59.0	34	45.98 ± 0.01	15
Heptadecyl octadecanoate	65.0—65.6	64.7	35	42.08 ± 0.01	20
Octadecyl octadecanoate	62	62	36	43.47 ± 0.02	18

[a] Found only in spectra taken from melt.
[b] Found only in spectra taken from acetone.

TABLE II
Relative Intensity of Orders of 001 (Long Spacing)

Name of compound	Relative intensities of orders of long spacing						
	1	2	3	4	5	6	7
Hexadecyl hexadecanoate	1000	35	550	15	196	10	63
Octadecyl tetradecanoate	1000	178	375	275	13	174	2
Heptadecyl tetradecanoate	1000	71	158	79	10	36	<4
Pentadecyl octadecanoate	1000	4	460	2	195	3	91
Heptadecyl hexadecanoate	1000	22	373	63	85	106	16
Hexadecyl tetradecanoate	1000	55	365	70	65	53	6
Hexadecyl octadecanoate	1000	36	496	16	196	11	55
Tetradecyl octadecanoate	1000	22	500	40	190	50	58
Octadecyl octadecanoate	1000	7	284	6	96	4	30

times from an acetone solution before x-ray diffraction patterns were taken.

Each compound was crystallized from the melt and also from acetone at room temperature. In most cases the same form was obtained by both procedures. X-ray diffraction measurements were made with a General Electric XRD-3 direct-recording unit, using nickel-filtered CUKα radiation ($\lambda = 1.5405$ Å), 1° beam slit, 0.1° detector slit, medium resolution sollar slit, scanning speed 0.2° per minute, chart speed 12 in. per hour, linear scale, 8-seconds time constant. The x-ray data listed in Table I were obtained from unground samples. Thin layers, measuring approximately 0.5 × 1.0 in., were firmly pressed on a glass slide to insure adherence. The long spacings listed in Table I are the averages for the number of orders listed in column 6; column 5 also gives the 95% confidence limits. The first and sometimes the second orders were often not included because of the limited accuracy with which they could be measured. Complete x-ray powder patterns are not, in general, included because of the space required.

RESULTS AND DISCUSSION

All the compounds investigated can be identified and distinguished by the x-ray diffraction powder data. Normally the long spacing alone is sufficient for identification. However examination of Table I reveals a few sets of compounds with almost identical long spacings: hexadecyl hexadecanoate and octadecyl tetradecanoate; pentadecyl octadecanoate, heptadecyl hexadecanoate (form with larger long spacing), and heptadecyl tetradecanoate (form with larger long spacing); hexadecyl tetradecanoate and hexadecyl octadecanoate; and tetradecyl octadecanoate and octadecyl octadecanoate. In these cases the compounds can be identified and distinguished by gross differences in the relative intensities of the long spacing orders (Table II). For example, examination of Table II shows that, for hexadecyl hexadecanoate, the fourth order is much weaker than the fifth order whereas for octadecyl tetradecanoate the reverse is true, thus permitting the two esters to be distinguished. Likewise the other sets of compounds can be distinguished by the relative intensities of their long spacing orders. For example, in the x-ray powder pattern of heptadecyl tetradecanoate, the fourth order is much stronger than the fifth order and the seventh order is missing; in pentadecyl octadecanoate the fourth order is much weaker than the fifth order and the seventh order is present and strong; and in heptadecyl hexadecanoate the fourth and fifth orders are about the same intensity and the seventh order is present, though not very intense. These three can also be distinguished by the formation of the second phase on crystallization. Pentadecyl octadecanoate does not form a second phase; heptadecyl hexadecanoate forms a second phase (long spacing 36.74 Å) from both melt and acetone; heptadecyl tetradecanoate forms a second phase (long spacing 37.83 Å) from acetone but not from melt. In the case of hexadecyl tetradecanoate and hexadecyl octadecanoate, the sixth order is stronger than the seventh order for the tetradecanoate whereas the reverse is true for the octadecanoate. In the case of tetradecyl octadecanoate and octadecyl octadecanoate, the sixth and seventh orders are about the same relative intensity for the tetradecyl derivative, but the sixth order is much weaker than the seventh order for the octadecyl derivative.

Several of the esters have long spacings which are a linear function of the number of carbon atoms in the molecule. Tetradecyl tetradecanoate, tetradecyl hexadecanoate, hexadecyl hexadecanoate, octadecyl tetradecanoate, hexadecyl octadecanoate, and octadecyl octadecanoate fall on a straight line when the long spacing (y) is plotted against the number of carbon atoms in each molecule (x) as shown in Fig. 1. The least-squares equation of this line is $y = (3.1440 \pm 0.9588) + (1.1205 \pm 0.0298)\,x$. This would indicate that these es-

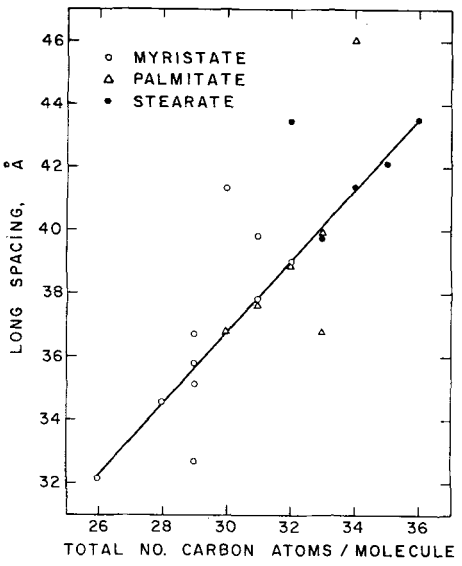

FIG. 1. Long spacing *versus* total number of carbon atoms per molecule for long-chain esters. Least-squares line derived from tetradecyl tetradecanoate, tetradecyl hexadecanoate, hexadecyl hexadecanoate, octadecyl tetradecanoate, hexadecyl octadecanoate, and octadecyl octadecanoate.

ters crystallize in the same polymorphic form. Therefore an attempt was made to index all reflections other than long spacings on the basis of the unit cell of hexadecyl hexadecanoate reported by Kohlhaas (4). It was assumed that all cell parameters remain constant except the c parameter, where the long spacing was taken as 0.5 c sin β. The attempt was successful, and the differences between observed and calculated values were within 0.01 Å. The characteristic side spacings seem to be the (110), (020), and (200) reflections in that order. It may be that the molecules lie on or near these planes.

A statistical analysis shows that the least-squares line of the above six compounds is indistinguishable from a least-squares line for the long spacing values of the ethyl esters of normal aliphatic acids (2, 3, 6). This would imply that the two series of compounds are isomorphous. Fortunately the unit cell of one of the ethyl esters is known, namely, ethyl stearate. The unit cell of ethyl stearate (8) seems to be isomorphous with that of hexadecyl hexadecanoate (4), but Kohlhaas reports the space group of hexadecyl hexadecanoate as $P2_1/a$ whereas Mathieson et al. report ethyl stearate as Ia. This would indicate that, although the unit cells are isomorphous, the structures are not.

If the long spacings of the six oxygen esters are compared with the values of the corresponding thiol esters (5), it is found that the long spacings of the corresponding sulfur compounds are greater on the average by 0.30 Å. This would be equivalent to an increase in the c parameter of about 0.68 Å. It may be assumed that the compounds are isomorphous and the same as hexadecyl hexadecanoate, that the covalent radii for oxygen and sulfur are those as given by Pauling (9), and that the aliphatic chain axis is roughly parallel to the c axis. If it is assumed that the asymmetric unit packs roughly as reported by Mathieson et al. (8), then the difference in the c axis between the corresponding sulfur and oxygen compounds should be about 1.18 Å. On the other hand, if it should be assumed that the asymmetric unit packs roughly, as suggested by Aleby for ethyl stearate (1), then the difference in c axis between corresponding sulfur and oxygen compounds would be about 0.50 Å. The observed 0.68 Å difference comes closer to the latter and would suggest that the compounds pack more nearly like the structure proposed by Aleby if the assumptions are correct. However, since the space group for ethyl stearate is different from the one reported for hexadecyl hexadecanoate, this conclusion is not certain without further study.

Normally an alternation in long spacing in a homologous series containing even and odd numbers of carbon atoms is typical of long-chain compounds (7). An examination of Fig. 1 seems to indicate that pentadecyl hexadecanoate, pentadecyl octadecanoate, heptadecyl hexadecanoate, and heptadecyl octadecanoate exist in the same polymorphic form. If their long spacing values are plotted against the total number of carbon atoms in each molecule (Fig. 1), it is seen that they do not fall in the same line with those containing an even number of carbon atoms but are slightly lower. Pentadecyl hexadecanoate, pentadecyl octadecanoate, and heptadecyl octadecanoate could be indexed by the same scheme as the evens, based on a unit cell of hexadecyl hexadecanoate. This would imply that the odd and even series are isomorphous. However the largest differences between observed and calculated values were greater than for the even series. The line representing the odd series would not necessarily be expected to coincide with that of the even series because of the difference in end packing between the two series. Further

work is required in order to clarify this point.

If the long spacing values of these odd-chained compounds are compared with the values of the corresponding thiol esters (5), on the average the sulfur compounds have long spacing values greater by 0.36 Å with one value outstandingly high, 0.47Å. With the even series, the average difference between the oxygen and sulfur compounds was 0.30 Å; one value was outstandingly low, 0.20 Å. If both these extreme values were eliminated, the averages would be 0.33 Å for the odds and 0.34 Å for the evens. Perhaps each class of oxygen compounds bears the same relationship to the corresponding class of sulfur compounds. This cannot be definitely established without further study.

A graph of the long spacing values of the ethyl esters and long-chain acids (B form) (2,3,6) against the total number of carbon atoms per molecule (Fig. 2) shows that the values fall on two straight lines, one for the even and one for the odd series, with the line for the odd series above that of the even series. This is the reverse of findings for esters of long-chain alcohols and thiols. Perhaps a change in packing occurs between the ethyl derivatives and the higher homologs in the odd series.

An examination of Table I and Fig. 1 shows that the compounds with odd numbers of carbon atoms tend to display more polymorphic forms for each compound than in the even series, both in the total number of different forms and forms for each individual compound. The evens tend to crystallize in only one form, whether crystallized from acetone or melt, but the odds tend to display several forms under the same conditions. The odds seem to display at least five different polymorphic forms, probably a sixth if the forms of pentadecyl tetradecanoate and heptadecyl tetradecanoate with the largest long spacings are also different. This difference is suggested by the fact that a straight line drawn between these two points would have a negative intercept at zero number of carbon atoms.

Fig. 1 suggests that the polymorphic forms of pentadecyl tetradecanoate and heptadecyl hexadecanoate which exhibit the smallest long spacing values are the same crystalline forms, but it cannot be said with certainty without further study. Fig. 1 also suggests that hexadecyl tetradecanoate, tetradecyl octadecanoate, and octadecyl hexadecanoate crystallize in the same polymorphic forms, where the aliphatic chain axis would be almost parallel to the c axis, but it cannot be established at this time without further study. Fig.1 shows that the form of heptadecyl tetradecanoate which displays the smaller long spacing value and one of the forms of pentadecyl tetradecanoate (second largest long spacing value) may crystallize in the same form as those isomorphous with hexadecyl hexadecanoate.

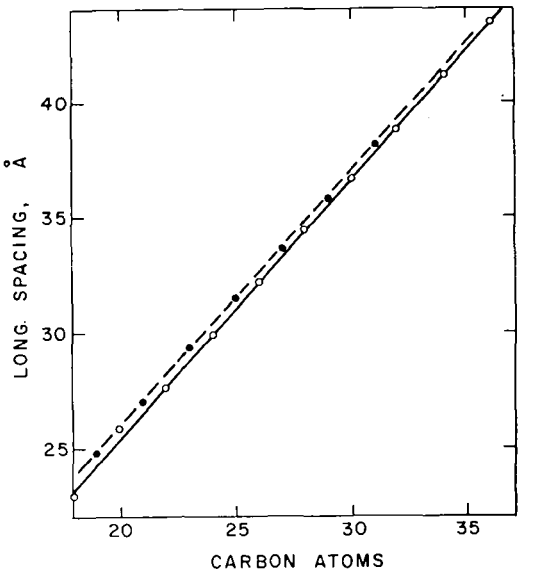

FIG. 2. Long spacing *versus* total number of carbon atoms per molecule, for ethyl esters of long-chain acids. Filled circles: Odd total number of carbon atoms. Open circles: Even total number of carbon atoms.

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The Lactone Precursor in Fresh Milk Fat: Identification of the Hydroxy Fatty Acids[1]

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ABSTRACT

A series of hydroxy fatty acids was identified from a polar glyceride milk-fat fraction. These acids were identified as trimethylsilyl (TMS) ether methyl ester derivatives. Identification was achieved by mass spectral analysis and comparison of gas chromatographic retention times with authentic compounds. Mass spectral fragmentation patterns of the TMS ether methyl esters were obtained, and correlation of important ions for structural identifications were made. In addition, semiquantitative data are presented.

INTRODUCTION

NATURALLY OCCURRING HYDROXY ACIDS have been the subject of recent investigations (1,2). In the last few years, knowledge of the chemistry of milk fat has progressed significantly, but all attempts to identify directly hydroxy fatty acids in milk fat have failed. Numerous lactones have been isolated from milk fat, ranging from C_8–C_{18} carbon atoms that included both saturated and unsaturated members (3,4,5). The logically suspected fatty acids, with the hydroxy group in the 4 and 5 position of the chain, are not stable; consequently the corresponding lactones are isolated.

This investigation was undertaken to determine the hydroxy fatty acid composition of milk fat.

EXPERIMENTAL PROCEDURE

The polar glyceride lactone precursor was prepared from fresh raw sweet cream, as described elsewhere (5). This fraction was subjected to BF_3 catalyzed methanolysis (6), and the methyl esters were separated into nonpolar and polar fractions by a liquid partition chromatographic procedure described by Frankel et al. (7). The hydroxy methyl esters in the polar fraction were converted to trimethyl silyl (TMS) derivatives (8) and extracted as described by Wood et al. (9).

Synthesis of Standard TMS Derivatives

Hydroxy acids with the hydroxy group in the 4 and 5 position are not readily available since cyclic esterification occurs rapidly. Therefore it was necessary to prepare standard reference compounds to assist in the identification of the unknown compounds isolated from milk fat. δ- and γ-Lactones are commercially available in high purity and can be hydrolyzed by alkaline solutions. Three ml of lactone were refluxed for 30 min with 10 ml of 1N NaOH, and after cooling the sample was lyophilized. The dried sodium salt of the hydroxy fatty acid was converted to the TMS derivative, as described above. Since the TMS ether linkage is labile to weakly acidic conditions (9), methyl esters were formed by esterification with diazomethane (10).

Analysis of the TMS Derivatives

The TMS derivatives were separated with an F & M Scientific Corporation Model 810 gas chromatograph, equipped with a hydrogen flame ionization detector. A 5-ft x ⅛-in. O.D. stainless steel column was packed with 20% Apiezon L on 100–120 mesh Celite 545. The column temperature was 230C. An inlet pressure of 30 psi was required for a helium flow through the column of 40 ml/min. The detector and injector port were maintained at 240C.

The effluent from the gas chromatograph was split; one portion passed to the flame ionization detector and the other to the heated EC–1 inlet of an Atlas MAT CH–4 Nier type mass spectrometer. Mass spectra were obtained by rapid (m/e 24–450 in 6.5 sec) magnetic scans of the chromatographic peaks and used for confirmation of retention data.

RESULTS AND DISCUSSION

Tentative identifications of the TMS derivatives from milk fat were made by comparison of retention times with a semilogarithmic plot of chain length and retention time obtained from authentic compounds. This line was

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TABLE I
Retention Data of TMS Ether Methyl Esters Isolated from the Lactone Precursor from Fresh Milk Fat

Compound	Retention time (minutes)	Authentic retention time (minutes)
5–TMS–Methyl octanoate	16.50	15.44[a]
4–TMS–Methyl decanoate	18.04	18.13
5–TMS–Methyl decanoate	18.78	18.17
4–TMS–Methyl undecanoate	.....	20.16
5–TMS–Methyl undecanoate	.....	20.08
5–TMS–Methyl dodecanoate	21.78	21.82
5–TMS–Methyl tetradecanoate	26.50	26.42[a]
5–TMS–Methyl pentadecanoate	27.88	29.26[a]
5–TMS–Methyl hexadecanoate	30.65	32.11[a]
5(?)–TMS–Methyl heptadecanoate	35.52	34.95[a]
5–TMS–Methyl octadecanoate	38.37	38.21[a]
5(?)–TMS–Methyl nonadecanoate	42.43	42.27[a]

[a] By extrapolation.

extrapolated to include compounds for which no standards were available. The retention data are shown in Table I.

In order to confirm these identifications by mass spectrometry, it was necessary to establish typical fragmentation patterns from the mass spectra of authentic compounds and to use this information to predict the spectra of those compounds for which there were no standards. The mass spectrum of authentic 5–TMS–methyl decanoate, shown in Figure 1, is a typical example. It should be noted that several of the fragments in Figure 1 are shown at 100% intensity. Analytical conditions were chosen to ensure maximal concentration of the GLC column effluent in the ion source so that the small fragments in the parent ion region of the spectra would be visible. Under these conditions the more prominent fragments were so intense as to be off scale, even on the least sensitive recorder galvanometer. Thus, although the proportions of the spectral fragment ions in this study are in no way quantitative, the pattern of the fragmentation can be extremely useful in structural elucidation.

Prominent ions of m/e 89 $[(CH_3)_3–Si–O]^+$, 73 $[(CH_3)_3–Si]^+$, 75 (rearrangement?), and 147 $[(CH_3)_3–Si–O–Si(CH_3)_2]^+$ (11) were observed in the spectra of all standard TMS derivatives and were extremely useful in distinguishing the TMS derivatives from normal milk fatty acid methyl esters. A peak at m/e 59, because of cleavage at the methyl ester moiety, was also ubiquitous.

Certain prominent fragments were helpful in establishing the structure of the TMS deriva-

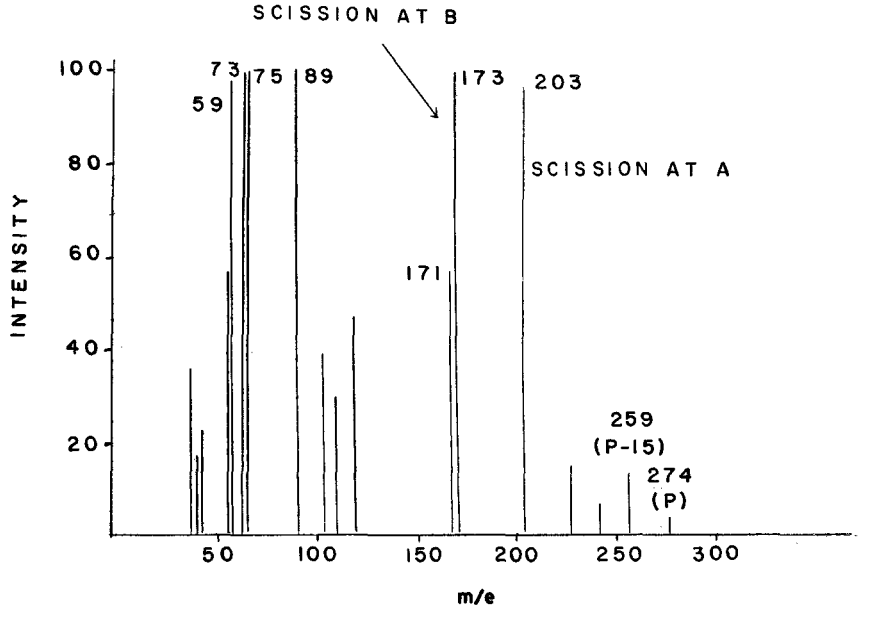


FIG. 1. The mass spectrum of 5-TMS methyl decanoate.

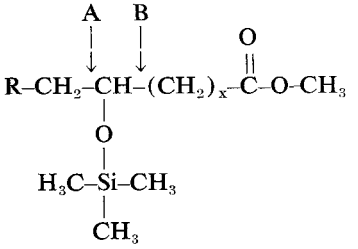
TABLE II
Major Fragmentation Ions (m/e) of Standard TMS Ether Methyl Esters

Compound	Fragment P	P–15	Scission at A	B
4–TMS–Methyl decanoate	274	259	189	187
5–TMS–Methyl decanoate	274	259	203	173
4–TMS–Methyl undecanoate	288	273	189	201
5–TMS–Methyl undecanoate	288	273	203	187
5–TMS–Methyl dodecanoate	302	287	203	201

TABLE III
Major Fragmentation Ions (m/e) Predicted for Certain TMS Ether Methyl Esters

Compound	Fragment P	P–15	Scission at A	B
5–TMS–Methyl octanoate	246	231	203	145
4–TMS–Methyl dodecanoate	302	287	189	215
5–TMS–Methyl tetradecanoate	330	315	203	229
5–TMS–Methyl pentadecanoate	344	329	203	243
5–TMS–Methyl hexadecanoate	358	343	203	257
5–TMS–Methyl heptadecanoate	372	357	203	271
5–TMS–Methyl octadecanoate	386	371	203	285
5–TMS–Methyl nonadecanoate	400	385	203	299

tives. The parent ion (P) is frequently weak or nonexistent; however P–15 is usually sufficiently prominent that an evaluation of the molecular weights can be made. The position of the TMS group can be readily established since prominent ions are found to result from fragmentation of the carbon chain on either side of the oxygenated carbon atom. Thus, in the diagram below, cleavage at A gives a strong fragment peak at m/e P–RCH_2 $[(CH_3)_3$–Si–O–$CH(CH_2)_x$–$COOCH_3]^+$. When x = 2 in the case of 4–hydroxy acids, this fragment appears at m/e 189; and when x = 3 as in 5–hydroxy acids, at m/e 203. This fragment mass establishes the position of the original OH group in relation to the carboxylic ester group. Fragmentation at B yields a prominent peak at P–$(CH_2)_x$–$COOCH_3$ or $[RCH_2CHO$–Si$(CH_3)_3]^+$. The mass of this fragment establishes the length of the R group. This fragment, with the molecular weight, is equally useful in establishing the position of the TMS moiety.



In addition, ions at m/e P–47 and P–31 were commonly observed. P–31 probably results from loss of $[CH_3$–$O]^+$ and P–47 could result from a rearrangement involving the TMS group. One possibility would be $[SiOH_3]^+$.

The fragmentation ions related to the structure of authentic TMS ether methyl esters are shown in Table II. On the basis of these patterns, the comparable ions were predicted for those compounds for which standards were not available. These predictions are shown in Table III.

The major fragment ions of compounds identified from milk fat as TMS ether methyl esters are shown in Table IV. These spectra were, in most cases, weaker than those of the standard compounds and were often contaminated by fragment ions from column bleed and from poorly resolved methyl esters of normal fatty acids. The fragments shown are those which were clearly prominent after subtracting the background spectra. Therefore, although many of the expected peaks do not appear in Table IV, sufficient mass spectral information is present for identification. The criteria for "positive identification" were coincidence of retention data and adequate mass spectral structural information. Compounds which did not fulfill both of these criteria were designated as being tentatively identified.

An approximation of the proportions of the various TMS-ether methyl esters was made from the peak areas of the respective GLC

TABLE IV
Major Fragmentation Ions (m/e) of Compounds Identified from Milk Fat as TMS Ether Methyl Esters

Compound	Source P	P–15	P–30	Scission at A	B	Other
5–TMS–Methyl octanoate[b]		231			145	89, 79, 75, 73
4–TMS–Methyl decanoate[a]		259		189	187	89, 79, 75, 73
5–TMS–Methyl decanoate[a]		259		203		201, 89, 75, 73, 59
5–TMS–Methyl dodecanoate[a]	302	287	272	203	201	89, 79, 75, 73
5–TMS–Methyl tetradecanoate[a]		315	300	203	229	89, 79, 75, 73
5–TMS–Methyl pentadecanoate[b]		329		203		201, 89, 79, 75, 73
5–TMS–Methyl hexadecanoate[a]		343		203		201, 103, 89, 79, 75, 73
5(?)–TMS–Methyl heptadecanoate[b]		357	342			89, 79, 75, 73
5–TMS–Methyl octadecanoate[a]		371		203		201, 103, 73, 75, 59
5(?)–TMS–Methyl nonadecanoate[b]	400	385				75, 73, 59

[a] Positive identification.
[b] Tentative identification.

peaks by assuming linear detector response by weight. The following distribution of hydroxy fatty acids was found in fresh milk fat in the indicated mole percentages: 5-hydroxy octanoic (8.0%), 4-hydroxy decanoic (9.5%), 5-hydroxy decanoic (10.9%), 5-hydroxy dodecanoic (13.7%), 5-hydroxy tetradecanoic (15.2%), 5-hydroxy pentadecanoic (4.8%), 5-hydroxy hexadecanoic (16.8%), and 5-hydroxy octadecanoic (17.4%). Hydroxy heptadecanoic (1.1%) and hydroxy nonadecanoic (2.6%) were also identified, but the position of the hydroxy groups could not be ascertained.

These results agree reasonably well with those of Jurriens and Oele, who reported slightly different proportions of C_{10} to C_{16} "bound lactones" in a sample of commercial butter (4). The finding of such a large quantity of 5-hydroxy octadecanoic acid is somewhat surprising, but the quantity correlates quite nicely with the recent finding of 18.7 mole % of δ-octadecalactone among the lactones produced from the same precursor (5).

No unsaturated hydroxy acids were found, even with an attempt to separate the polar methyl ester fraction by silver nitrate-impregnated silicic acid thin-layer chromatography.

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Quantitative and Qualitative Lipid Correlation in Experimental Endogenous Hyperlipemia

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ABSTRACT

The reversible endogenous hyperlipemia in dogs, elicited by the detergent Triton which was given intravenously, was used to study the interrelations of serum lipids. In the cholesterol ester fraction an increase occurs in both monounsaturated and in saturated fatty acids, excepting myristic; while a decrease occurs in polyunsaturated fatty acids. The fatty acids of cholesterol esters of normal dogs contain 22% oleic acid, and only 24% when serum lipids are increased to almost double their normal value (TC=400-500 mg/100 ml). However there is a critical level above which a rapid rise in oleic acid occurs and, in severe hyperlipemia (TC=1500 ±430 mg/100 ml), this acid constitutes almost half of the esterified fatty acid component.

Since there is no evidence that Triton directly regulates fatty acid synthesis, the lipid fraction-fatty acid interrelationship may be secondary to lipid mobilization from endogenous sources. This concept is supported by the fact that the increased serum fatty acids are only those which can be synthesized by animals. It is suggested, on the basis of a marked increase of endogenously produced fatty acids, that, at critical lipid levels, shortage of polyunsaturated fatty acids from exogenous sources occurs. This might be of sufficient degree to accelerate fatty acid synthesis to meet the need for fatty acids for energy requirements. There may also be need of fatty acid for esterification of chiefly the accumulated free cholesterol split from lipoprotein by Triton.

Triton-induced changes in cholesterol ester fatty acids result in patterns which closely resemble those in the adipose tissue of dog and man and in the serum of human endogenous hyperlipemia.

INTRODUCTION

BOTH CLINICAL (1–5) AND EXPERIMENTAL (6,7) HYPERLIPEMIA suggest a correlation between the levels of serum or tissue cholesterol and the degree of saturation of the fatty acids esterified with cholesterol ester.

With the increase in hyperlipemia that resulted from the detergent Triton-injection in this study, saturated and monounsaturated fatty acids greatly increased (almost doubled) at the expense of polyunsaturated FA. The evidence suggests that the increased serum cholesterol ester fatty acids are only those which can be synthesized by animals. The changed serum fatty acid patterns in hyperlipemia because of Triton are similar to FA patterns in the adipose tissue of normal dogs (7) and to serum FA patterns in spontaneous hyperlipemia in man (5). Triton hyperlipemia in dogs may resemble that of human endogenous hyperlipemia, thus providing an experimental model for the investigation of correlative relationships among lipids inherent in the nature of endogenous hyperlipemia.

METHODS

Hyperlipemia was produced according to a slight modification of the method by Scanu et al. (8). Five male and five female mongrel dogs received 100 to 200 mg/kg body weight of Triton WR–1339 (Winthrop Laboratories, New York, N. Y.), dissolved one to five in a phosphate buffer (pH 7.2) solution and injected intravenously twice a week for a period of six to eight months. For the base-line lipid values, two blood samples were drawn from each animal during four weeks prior to Triton injection. Biweekly samples of blood were taken during the control and experimental period and were analyzed for serum lipids by extracting of 1 ml of serum with 25 ml of chloroform-methanol mixture (2:1, v/v) at room temperature. The organic extract was washed with 8 ml of 0.02% calcium chloride solution. On standing at 4C overnight, a two-phase system was produced. The chloroform phase was concentrated by evaporation in vacuum or under nitrogen. Nonpolar lipids were separated from polar lipids on a 7-mm silicic acid column. The column was prepared by using 800 mg of 100-200 mesh silicic acid, which was suspended in chloroform, and the suspension was transferred to the column. The

packed column was washed with methanol, then with chloroform. Lipid was applied in chloroform and eluted with 25 ml of chloroform, followed by 25 ml of methanol. Total cholesterol (TC), free cholesterol (FC), and triglycerides (TG) from the chloroform phase and phospholipids (PL) from the methanol phase were determined as in the earlier study (9). In addition, extracted serum lipids were separated on thin-layer chromatography, using silica gel G as an adsorbent.

The slurry was made by using equal parts of silical gel and 0.01% of rhodamine B solution (w/v) and a 0.4–mm layer applied to 8 by 8–in. glass plates. Cholesterol ester, triglyceride, and phospholipid fractions were separated by using petroleum ether:diethyl ether:acetic acid (90:10:1) solvent system. Lipid fractions were identified under ultraviolet light and transmethylated in the presence of silica gel in 5% sulfuric acid in methanol and a few crystals of hydroquinone for two hours. Methyl esters were extracted with petroleum ether and dried with sodium sulfate:sodium bicarbonate (4:1). The solvent was evaporated under nitrogen, and the residue was redissolved in hexane for analysis by gas-liquid chromatography. Completeness of transmethylation and quantitative recovery of methyl esters was verified. F & M model 810 with flame ionization detector was used isothermally at 185C. Helium, at a flow rate of 60 ml/min, was the carrier gas. A column ¼ in. in diameter and 6 ft long was packed with 13% ethylene glycol succinate on 80–100 mesh Gas–Chrom P (Applied Science Laboratories Inc., State College, Pa.). The peak area was calculated by using a Disc integrator, and the results were rechecked by triangulation. On the basis of these standards obtained commercially, detector response was calibrated for individual fatty acids; correction factors, when necessary, were applied. Column separation was checked with methyl ester mixtures supplied by the National Institutes of Health. Duplicate chromatograms were run on each sample.

RESULTS

The effects of periodic Triton injection on lipid mobilization and transport in serum are evaluated in terms of three categories.

Changes in Lipid Fractions and Cholesterol Ester Fatty Acid Percentage Composition

With the injection of Triton all animals developed hyperlipemia during the eight-month study period. In general, the degree of hyperlipemia was proportional to the dosage and/or to the length of administration of Triton. With the increase in lipid fractions, there was a change in the fatty acid percentage composition of the cholesterol ester fraction. Total cholesterol varied from a low of 172 ± 20.7 mg/100 ml in the normal (pre-Triton period)

TABLE I

Averages and Standard Deviations of Serum Lipid Fractions in mg/100 ml and Serum Cholesterol Ester Fatty Acids in Percentage Composition at Increasing Levels of Total Cholesterol, Resulting from Triton-Induced Lipemia

(Two biweekly blood samples prior to Triton injection, then biweekly samples during six to eight months of twice-a-week Triton injection: Five male and five female dogs. Sample size = 183)

Triton-lipemia stages	Pre-Triton or normal	Early	Early	Moderate	High
Total cholesterol levels in mg/100 ml	200	200–299	300–499	500–999	>1000
Sample size (n)	10	21	46	48	58
Averages and standard deviations	Av. S.D.	Av. S.D.	Av. S.D.	Av. S.D.	Av. S.D.
Lipid Fractions					
Total cholesterol (TC)	172.0 ± 20.7	255.0 ± 25.8	412.0 ± 55.6	701.0 ± 142.0	1577.0 ± 430.0
Free cholesterol (FC)	42.0 ± 12.1	68.0 ± 17.2	123.0 ± 55.5	355.0 ± 222.0	1181.0 ± 376.0
Phospholipid (PL)	316.0 ± 38.1	372.0 ± 71.0	485.0 ± 131.0	925.0 ± 355.0	2204.0 ± 534.0
Triglyceride (TG)	42.0 ± 9.1	59.0 ± 37.3	109.0 ± 105.0	711.0 ± 702.0	2934.0 ± 1664.0
Fatty Acid Profile, Cholesterol Esters					
Myristic (14:0)	0.2 ± 0.1	0.1 ± 0.01	0.3 ± 0.3	0.3 ± 0.7	0.4 ± 0.5
Palmitic (16:0)	11.4 ± 1.3	11.1 ± 1.9	11.1 ± 2.3	11.7 ± 2.9	15.8 ± 2.0
Palmitoleic (16:1)	1.8 ± 0.7	3.1 ± 0.7	3.0 ± 0.6	3.7 ± 1.6	5.1 ± 1.0
Stearic (18:0)	0.6 ± 0.3	0.9 ± 0.9	1.0 ± 1.2	2.9 ± 2.8	8.5 ± 2.7
Oleic (18:1)	24.3 ± 2.1	25.6 ± 3.2	25.1 ± 4.6	32.3 ± 13.1	44.7 ± 6.3
Linoleic (18:2)	47.1 ± 5.3	48.0 ± 7.9	45.0 ± 5.9	36.8 ± 10.9	21.4 ± 7.3
Arachidonic (20:4)	14.3 ± 3.8	11.1 ± 4.3	14.8 ± 4.6	12.2 ± 7.6	3.9 ± 3.0

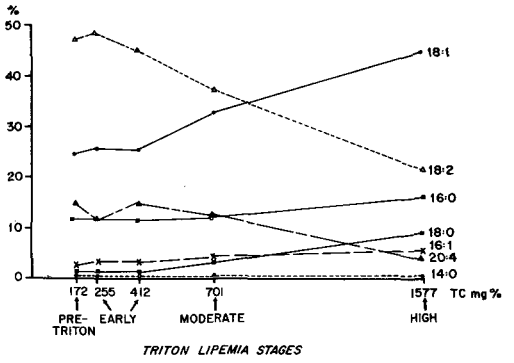

FIG. 1. Average CE fatty acid percentage composition at increasing levels of total cholesterol, resulting from Triton-induced lipemia.

to a high of 1577 ± 430.0 mg/100 ml in the late stages of Triton treatment (Table I). Triglyceride varied from 42 ± 9.1 to 2934 ± 1664 mg/100 ml respectively.

Fatty acid composition of the cholesterol ester fraction was significantly different in severely hyperlipemic sera as compared with normal (pre-Triton period) or even moderately lipemic sera. In those animals with severe hyperlipemia the saturated and monounsaturated FA represented a higher percentage in the total FA whereas, in the pre- and early stages of Triton-induced lipemia, the polyunsaturated FA made up a higher percentage of the total FA. Table I and Figure 1 contain data from five stages of the development of Triton-induced hyperlipemia. The most noticeable changes were linoleic and oleic acid compositions. In the pre- and early Triton treatment stage, when TC values were low (172 ± 20.7 mg/100 ml), linoleic acid constituted almost half (47.1 ± 5.3%) of all FA present in the CE fraction. At moderate hyperlipemia (TC level of 701 ± 142 mg/100 ml) 36.8% of linoleic acid was still present, but at severe hyperlipemia (TC values of 1577 ± 430 mg/100 ml) only 21.4% of the same acid remained. For the corresponding total cholesterol classification, the percentage composition of oleic acid was 24.3, 25.1, and 44.7% respectively. However myristic acid did not change, and the percentage of palmitoleic increased only in extreme hyperlipemia (TC over 1000 mg/100 ml).

Correlation Matrix of Lipid Fractions and Cholesterol Ester Fatty Acids

The degree of association, measured by the coefficients of correlation, among lipid fractions is very high (Table II). TC correlates to the extent of 0.97 with TC 0.97 with PL and 0.84 with TG. All correlations are positive, a result which signifies that high values of one lipid fraction are accompanied by high values of the other lipid fractions. In general, the degree of association among fatty acids is not as high as among the lipid fractions. There is a high negative association (–0.93) between linoleic and oleic acid, and a fairly high positive association (0.80) between stearic and oleic acid. Also myristic acid is not associated with any fatty acid except palmitoleic, but this association (0.27) is numerically small. There is also a fairly high degree of correlation between a cholesterol ester fatty acid and a lipid fraction. All CEFA yield similar correlations with total cholesterol, free cholesterol, phospholipid, and triglyceride.

Correlation Matrix of Lipid Fractions and Triglyceride or Phospholipid Fatty Acids

The degree of association, measured by the coefficients of correlation, among lipid frac-

TABLE II

Correlation Matrix of Cholesterol Ester Fatty Acids (CEFA) and Lipid Fractions[a]

14:0	16:0	16:1	18:0	18:1	18:2	20:4	TC	FC	PL	TG
14:0	.....[b]	0.27	.....	.....	.....	.....	.....	.....	.....	.....
16:0		0.75	0.82	0.68	–0.78	–0.74	0.66	0.70	0.72	0.72
16:1			0.71	0.70	–0.74	–0.65	0.59	0.64	0.64	0.60
18:0				0.80	–0.88	–0.77	0.83	0.84	0.87	0.80
18:1					–0.93	–0.82	0.70	0.74	0.75	0.71
18:2						–0.64	–0.77	–0.79	–0.81	–0.76
20:4							–0.63	–0.69	–0.70	–0.67
TC								0.97	0.97	0.84
FC									0.97	0.84
PL										0.84

[a] $r > 0.17$ and $r < -0.17$ are significant at 1% level, and if $(1 + r_1)(1 - r_2)/(1 + r_2)(1 - r_2)$ is greater than 1.42 and 1.63, then the difference between r_1 and r_2 is significant at the 5% and 1% levels respectively ($N = 183$).

[b] Nonsignificant r's denoted by .....

TABLE III

Correlation Matrix of Triglyceride Fatty Acids (TGFA) and Lipid Fractions[a]

	16:0	16:1	18:0	18:1	18:2	20:4	TC	FC	PL	TG
14:0	.....[b]	0.41	−0.28	−0.29	.....	.....	.....	.....	.....	.....
16:0		−0.49	0.38	−0.29	−0.29	−0.26	0.36	0.35	0.38	0.33
16:1			−0.62	.....	0.35	.....	−0.47	−0.51	−0.51	−0.44
18:0				.....	−0.46	.....	0.61	0.65	0.66	0.55
18:1					.....	−0.43	.....	.....	.....	.....
18:2						.....	.....	−0.26	.....	.....
20:4							.....	.....	.....	.....

[a] $r > 0.25$ and $r < -0.25$ are significant at the 1% level, and if $(1 + r_1)(1 - r_2)/(1 + r_2)(1 - r_1)$ is greater than 1.4 and 1.63, then the difference between r_1 and r_2 is significant at the 5% and 1% levels respectively ($N = 100$).

[b] Nonsignificant r's denoted by ..... The correlations among the lipid fractions is the same as in Table II.

tions and fatty acids esterified with triglyceride (Table III) and phospholipids (Table IV) in the Triton-induced hyperlipemia is not as high as among cholesterol ester fatty acids.

With the increase in lipid fractions the change in the fatty acid percentage composition of triglyceride and phospholipid fractions was not as marked as in the cholesterol ester fraction. $R^2 \times 100$ ("percentage of association") is 71% for fatty acids esterified with cholesterol, only 35% for fatty acids esterified with triglyceride, and 47% for fatty acids esterified with phospholipids. Because of this low correlation among lipids and FA esterified with TG and PL, these FA were excluded from further evaluation.

DISCUSSION

Scanu et al. (10) demonstrated in vitro that, after the addition of Triton to lipoproteins and chylomicrons, the detergent combines preferentially with their lipid moiety, weakening the association between protein and lipid and displacing part of the protein moiety from chylomicrons. Scanu and Page (11), from data in dogs treated with Triton and injected with labeled cholesterol and phospholipids, suggested that these split lipids are not locked in circulation but that their rise in plasma is secondary to an imbalance between their rate of synthesis and their rate of removal. The synthesis exceeds the removal.

The data show a slow rise of lipids for one to three months, which was accompanied with only a minor change in fatty acid composition. However, when serum lipid levels are almost double their normal value (total cholesterol = 400–500 mg/100 ml), a more rapid increase occurred in the cholesterol ester fraction in saturated, excepting myristic, and in monounsaturated along with a decrease in polyunsaturated fatty acids. The fatty acids of cholesterol esters of normal dogs contained 22% oleic acid, and only 24% when serum lipids were almost double their normal value. In severe hyperlipemia (total cholesterol = 1500 ± 430 mg/100 ml) oleic acid constituted almost half of the esterified fatty acids. The corresponding amounts of linoleic acid were 47, 37, and 21% respectively under these experimental conditions.

It was first established by Page and van Slyke in 1935 (12) and later confirmed by several other investigators that lipid fractions (classes) and their fatty acid percentage composition in serum (1) and in the tissue of man

TABLE IV

Correlation Matrix of Phospholipid Fatty Acids (PLFA) and Lipid Fractions[a]

	16:0	16:1	18:0	18:1	18:2	20:4	TC	FC	PL	TG
14:0	.....[b]	0.30	−0.44	.....	.....	.....	.....	.....	.....	.....
16:0		0.32	−0.41	0.32	0.33	−0.48	0.53	0.56	0.55	0.45
16:1			.....	.....	.....	.....	0.39	0.41	0.40	0.39
18:0				.....	−0.70	.....	−0.30	−0.33	−0.35	−0.33
18:1					.....	−0.76	0.36	0.40	0.35	0.37
18:2						.....	0.29	0.32	0.35	0.35
20:4							−0.44	−0.47	−0.44	−0.40

[a] $r > 0.25$ and $r < -0.25$ are significant at the 1% level, and if $(1 + r_1)(1 - r_2)/(1 + r_2)(1 - r_1)$ is greater than 1.4 and 1.63, then the difference between r_1 and r_2 is significant at the 5% and 1% levels respectively ($N = 100$).

[b] Nonsignificant r's denoted by ..... The correlations among the lipid fractions is the same as in Table II.

(13) and animal (14,9) in health have characteristic patterns. These appear to be maintained in dynamic equilibrium and are not easily disturbed even by continuous Triton injection. Only after lipids accumulate in serum to approximately double their normal levels does the shortage of long-chain FA become pronounced enough to upset the equilibrium and cause a significant change in fatty acid patterns.

The intercorrelations of cholesterol ester fatty acids and serum lipid fractions in this study (Figure 1 and Table II) support the concept of dynamic equilibrium of this system. In the data there is a positive correlation of lipids with 18:0, 18:1, 16:0, and 16:1. There is a negative correlation with 18:2 and 20:4 and, because each correlation is significant, it is concluded that each FA, except myristic, by itself is important from a correlative point of view.

There is no direct evidence that Triton regulates fatty acid synthesis. The lipid fraction-fatty acid interrelationship therefore may be secondary to lipid mobilization from endogenous sources. The body has a limited ability to synthesize polyunsaturated although it can readily produce saturated and monounsaturated fatty acids (15–17). Therefore, during the rapid development of Triton hyperlipemia, a relative deficiency of polyunsaturated fatty acids occurs. All fatty acids capable of being synthesized by the animal (16:0), 16:1, 18:0, and 18:1 except 14:0, increase in cholesterol ester fraction; essential fatty acids (18:2 and 20:4), which are mainly derived from exogenous sources, are decreased. The endogenously produced fatty acids may esterify in serum with accumulated lipid (split from lipoprotein by Triton). The esterification of sterol is necessary for complexing it with lipoprotein for transport (18). Thus a change in cholesterol ester fatty acid patterns occurs: an increase in monounsaturated and saturated fatty acids except myristic (14:0) and a decrease in polyunsaturated fatty acids. The cleavage of lipid by Triton from the vehicular lipoprotein and its removal through reticuloendothelial cells (7,8) creates lipid shortage. This shortage is more apparent when lipids in serum are almost double their normal value. At this critical point a more sudden rise in lipid and a rapid change in fatty acid patterns occur (Table II).

With the onset of Triton hyperlipemia, typical fatty acid patterns develop which closely resemble those in the adipose tissue of dog (7) and man (13). With the mobilization of lipids from endogenous sources the changes in fatty acid patterns are also somewhat similar to those in spontaneous hyperlipemia in serum as described by Schrade (1), Fletcher (3), and Warembourg (5).

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Determination of the Complete Structure of Natural Lecithins[1]

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ABSTRACT

A method is described for the separation, identification, and quantitative estimation of the individual molecular species occurring in natural lecithin mixtures. Purified lecithin preparations are converted into diglyceride acetates by enzymic dephosphorylation and acetylation. The diglyceride acetates are separated on the basis of the degree of unsaturation and the molecular geometry by means of chromatography on thin layers of silica gel which are impregnated with silver nitrate. The various acetates thus resolved are separately recovered from the plates and diluted with tridecanoin internal standard; the quantitative distribution of the molecular weights is determined by gas chromatography.

Suitable aliquots of the saturated and unsaturated diglyceride acetates are further analyzed for over-all and for positional distribution of fatty acids. The identity and proportions of the various lecithins are deduced by integration and normalization of all the experimental data. Where doubt exists, specific diglyceride acetates are isolated by preparative gas chromatography, and their fatty acid composition is determined. The method is illustrated with data obtained for the mixed lecithins of egg yolk. The general approach is applicable to the determination of the structure of other phospholipids of comparable complexity.

INTRODUCTION

THE NATURAL LECITHINS contain several different fatty acids and represent a complex mixture of various molecular species. Although a specific positional placement of the fatty acids within the lecithin molecule has been recognized (1), the true composition of any natural lecithin mixtures has never been determined.

Renkonen (2) has demonstrated that a rather detailed description of many phosphatides can be obtained by an investigation of the diglyceride parts of these molecules by the thin-layer chromatographic methods employed in triglyceride analyses. The present study confirms this observation and demonstrates that the inclusion of a gas chromatographic analysis of the diglyceride acetates in the procedure permits a complete determination of the structure of natural glycerophosphatides.

The diglyceride acetates however must be prepared by hydrolysis with specific phospholipases and acetylation. Direct conversion of lecithins into the diglyceride acetates by acetolysis has been shown (3, 4) to result in a partial migration of the acyl groups. Fractionation of unmodified lecithins by selective argentation (5) has not been sufficiently refined for accurate reproducibility. Both of these techniques however may serve as analytical aids in specific cases.

MATERIALS AND METHODS

All solvents and reagents were of Fisher Certified Reagent-grade purity and were tested prior to use for lipid contaminants. The test consisted of evaporating 25-50 ml of the solvent, or a chloroform extract of the reagent to dryness, and injecting a chloroform solution of the residue into the gas chromatograph under the working conditions. All containers were rinsed prior to use with chloroform: methanol (3:1). Phospholipase C (α-toxin of *Clostridium welchii)* and pancreatic lipase (Steapsin) were purchased from the Sigma Chemical Company, St. Louis, Mo. Both enzymes were allowed to autodigest for about 30 min under the incubation conditions, and the digests were exhaustively extracted with diethyl ether prior to the addition of the substrate.

Preparation of Egg Yolk Lecithins

The mixed lecithins were prepared from fresh eggs of White Leghorn hens as follows. An egg was carefully broken, the white poured off, and the yolk, while still in the sac, washed with 0.9% NaCl. After removing the sac with forceps, the yolk was homogenized with 100 ml of saline and 100 ml of chloroform: meth-

[1]Presented in part at the AOCS Meeting in Philadelphia, October 1966.

anol (2:1), and the mixture was centrifuged to separate the phases. The total egg yolk lipid (7-10 g) was isolated from the lower phase in the usual manner. It was finally dissolved in 10 ml of petroleum ether and stored at –20C in a tightly closed screw-cap vial.

The lecithins were isolated as required, from the total lipid extract, by preparative thin-layer chromatography on silica gel H (Merck and Company), using 0.5-mm thick layers and 20 x 20-cm plates (DeSaga Equipment Company). About 400-500 mg of total lipid were applied as a band to each plate, and the plates were developed twice with a mixture of chloroform:methanol:water (65:25:4, v/v/v). Between developments the plates were allowed to dry by removing them from the developing tank for about one minute. No acid was added to the system as it greatly prolonged the drying time of the plate after the run, caused overlapping of the lysolecithins and sphingomyelins with the lecithins, and upset the pH of the buffer mixture used in the subsequent enzyme digestion. The lipid bands were located by spraying the plate with a minimum of a solution of 0.05% 2, 7-dichlorofluorescein in 50% methanol and viewing the plate under ultraviolet light. The lecithins separated in this way appeared free of the impurities which are readily demonstrated in other solvent systems that are commonly used for the resolution of phospholipids on thin-layer plates.

Preparation of Diglyceride Acetates

For the preparation of the free diglycerides, the lecithins were recovered from the plate together with the silica gel and were dropped directly into the enzyme solution. About 50 ml of the buffered phospholipase C of Renkonen (2) were used to digest completely the lecithins from 2–3 thin-layer plates. The incubations were performed for 30–60 min at 28–30C under diethyl ether. At the end of the digestion the phases were separated, and the extraction was completed with several small portions of fresh ether. The diglycerides were immediately purified by chromatography on silica gel H (20 x 20-cm plates, 0.25–mm thick layers), using petroleum ether:diethyl ether:formic acid (60:20:1.5, v/v/v) as the developing solvents. The diglyceride bands were located by spraying the plates with the fluorescein solution and viewing under ultra-violet light. The pure diglycerides were recovered by elution with chloroform. The diglycerides were dissolved in about 0.1 ml of dry pyridine (distilled over BaO) and were treated with 0.25–0.5 ml of acetic anhydride at room temperature (12 hours). The acetates were recovered after evaporation of the excess reagents under nitrogen. The diglyceride acetates (Rf 0.45) were purified by thin-layer chromatography, using the conditions just outlined for the preparation of the diglycerides. *The purification steps are absolutely essential since traces of mono-, di- or triglycerides remaining in the acetate mixture inevitably result in a contamination of the diglyceride acetate bands recovered from the silver nitrate plates.*

Separation of Diglyceride Acetates

The separation of the diglyceride acetates, on the basis of the number of double bonds per molecule, was performed on silica gel G (Merck and Company) plates (20 x 20 cm, 0.25–mm thick layers) containing 20% silver nitrate. The material (10–25 mg) was applied as a band about 2.5 cm from the edge of the plate, and the plate was developed twice in the same direction with a solution of 0.7 or 0.8% methanol in chloroform. The diglyceride acetate bands were located by spraying with the solution of fluorescein in aqueous methanol. The individual bands were recovered from the plate by elution with chloroform:methanol (9:1), to which 5% water was occasionally added to check on possible incomplete recovery of the more unsaturated components. The eluates were reduced to dryness under nitrogen, dissolved in petroleum ether, and immediately examined by gas chromatography.

Positional Analysis of Fatty Acids

The positional distribution of the fatty acids in the lecithin molecules was determined by pancreatic lipase hydrolysis of the diglyceride acetates. The hydrolysis was performed under the general conditions of Luddy et al. (6) except that the reaction mixture was saturated with diethyl ether and that the substrate occasionally was added together with the silver nitrate and the silica gel. Five to fifty milligrams of the total diglyceride acetate, or of the diglyceride acetates of specific degree of unsaturation, were incubated at one time. After 10 min of digestion the solution was acidified and extracted with diethyl ether. The ether extracts were chromatographed on thin-layer plates, as described for the diglycerides. The free fatty acids and the monoglycerides were recovered separately, and the component acids were determined by gas chromatography. The positional distribution of the fatty acids in the original lecithin mixture was determined by hydrolysis with phospholipase A (7).

Gas Chromatography

Gas chromatographic analyses of fatty acids were performed on the F & M High Efficiency Gas Chromatograph, Model 402. The fatty acid methyl esters were prepared by transmethylating the lecithin or the diglyceride acetate mixtures with 10% (w/v) H_2SO_4 in methanol at 80C for 10 hr in polyethylene-sealed screw-cap vials. The methyl esters were recovered by extraction with petroleum ether. The chromatographic separations were performed isothermally at 190C and at 220C by using glass columns (4 ft x ⅛ in. ID) packed with 10% EGGS-X on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College, Pa.). The peak identity and the quantitative estimates of the long-chain esters were confirmed by chromatography on a column containing 5% SE-30 on 100–120 mesh Gas-Chrom Q, using temperature programming (150-250C, 4C/min). Quantitative results with National Heart Institute Fatty Acid Standards (Mixtures D, E, and F) agreed with the stated composition data, showing a relative error less than 1% for major components (more than 10% of total mixture) and less than 5% for minor components (less than 10% of total mixture).

Gas chromatographic separations of the diglyceride acetates were performed on a Beckman GC-4 Gas Chromatograph with a specially modified on-column injector heater (8). The instrument was equipped with a differential electrometer and was operated with parallel columns. These were stainless steel tubes (⅛ in. O. D. x 2 ft) and were packed with 3% JXR or 1% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories Inc.). The recordings were made on a Beckman 10-in. Potentiometric 1 mv recorder, equipped with a disc integrator. One to five μl of 0.5–1% (w/v) solutions of diglyceride acetate in chloroform were injected. Quantitative results with standard mixtures (made up of trilaurin, trimyristin, tripalmitin, and tristearin) agreed with the weight proportions, showing a relative error less than 2% for glycerides up to tripalmitin and less than 10% for tristearin.

Preparative gas chromatography of the diglyceride acetates was done on the Aerograph Autoprep 700, using the conditions described for collection of short-chain triglycerides (9). This instrument was equipped with a stream splitter and a hydrogen flame ionization kit.

Scheme of Analysis

A purified lecithin sample of known fatty acid composition is subjected to hydrolysis by phospholipase C. The released diglycerides are collected, purified, and acetylated. Part of the acetate mixture is injected into the gas chromatograph, and the over-all molecular-weight distribution is determined. The rest of the sample is segregated on the basis of unsaturation on thin-layer plates of silica gel which are impregnated with silver nitrate. The separately recovered diglyceride acetates of uniform geometry and the degree of unsaturation are examined in the gas chromatograph for molecular-weight distribution and for proportional contributions, using tridecanoin as internal standard. The various diglyceride acetates are finally analyzed for over-all composition and for positional placement of the fatty acids. The structure of the original lecithin mixture is then derived from these data.

RESULTS AND DISCUSSION

The general method of analysis is sufficiently flexible to permit an amplification of any of the analytical steps and inclusion of new ones if additional information is required. For example, specific diglyceride acetate peaks can be collected in the preparative gas chromatograph and the component fatty acids identified. Furthermore the system permits numerous cross-checks for correcting the recoveries of fatty acids and the diglyceride acetates. Hydrolysis of the original lecithin mixture by phospholipase A allows an independent verification of the positional distribution of the fatty acids derived from the lipase digestions of the diglyceride acetates. Finally a judicious selection of enzymes should permit the application of the technique to the determination of the structure of other phosphatides of comparable complexity.

The total fatty acid composition of the diglyceride acetates was usually identical to that of the original lecithin, and the over-all proportions of the acids in the β-position of the diglyceride acetates were similar to those of the acids released from the original lecithin by phospholipase A. Occasionally, however, lower levels of the more unsaturated long-chain acids were found in the diglyceride acetates. Table I shows the fatty acid composition of a representative sample of egg yolk lecithins and a comparison of the present values with those recorded by previous investigators (4,10). Every lecithin preparation contained small amounts of C_{20} and C_{22} unsaturated acids, as did

TABLE I
Fatty Acid Composition of Total Egg Yolk Lecithins
(Mole, %)

Fatty acids	Original			Reconstituted			Literature	
	Total[a]	α[b]	β[b]	Total	α	β	Total[c]	β[d]
14:0	0.2	0.5		0.2	0.5			
16:0	31.6	61.3	1.6	33.4	65.7	1.0	33.0	0.9
16:1	2.1	1.1	1.2	1.5	1.4	1.5	2.1	0.7
18:0	12.3	27.0	0.1	13.0	25.7	0.1	15.4	
18:1	30.2	8.5	52.1	29.0	5.7	52.8	31.7	55.1
18:2	16.2	1.2	33.2	16.4	0.8	33.0	17.8	34.8
18:3	0.1		0.1			0.1		
20:0	0.1	0.2		0.1	0.1			
20:1	0.1	0.1	0.1	0.1	0.1	0.1		
20:3	0.4		0.1	0.3		0.5		
20:4	3.8		6.5	3.2		6.3		8.5
22:3	0.3		0.5	0.1		0.1		
22:4	0.6		1.0	0.6		1.2		
22:5	0.1		0.1	0.1		0.3		
22:6	1.9		3.5	2.0		4.0		

[a] Fatty acids of original egg yolk lecithins.
[b] Fatty acids of diglyceride acetates as determined by pancreatic lipase hydrolyses.
[c] Data of Blank *et al.* (10).
[d] Data of Nutter and Privett (4).

Renkonen's (2). Essentially all of these acids, together with the 18:2 and large amounts of 18:1 acid, were preferentially attached to the β-position, where only small amounts of palmitic acid could be shown to occur. The selectivity in the placement of the C_{16}, C_{18}, and C_{20} fatty acids compares well with that noted for egg yolk lecithins by Nutter and Privett (4) and by Renkonen (2). Table I also records the fatty acid compositions calculated for the reconstituted lecithin mixture. These latter values are in excellent agreement with the original data.

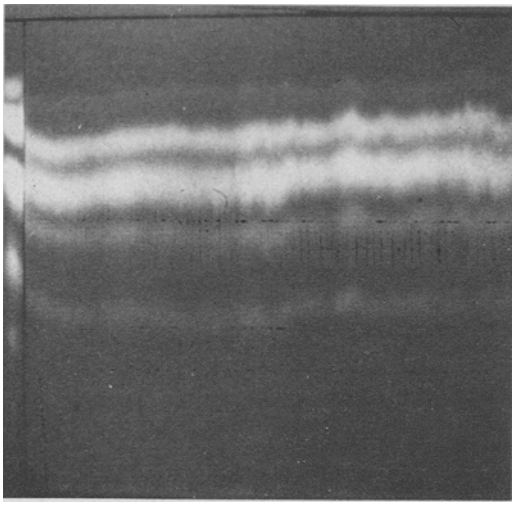

FIG. 1. Silver nitrate TLC of diglyceride acetates of egg yolk lecithins. Left lane, randomized mixture of triolein and trimyristin, representing (from top to bottom) saturates, monoenes, dienes, trienes, and diolein. Right lane, diglyceride acetates of egg yolk lecithins, representing (from top to bottom) saturates, monoenes, dienes, trienes I, and trienes II. Hexaenes are retained at the origin. Tetraenes and pentaenes do not show up well in this print. Chromatography conditions are given under Materials and Methods.

Figure 1 shows a thin-layer chromatoplate of the diglyceride acetates on silica gel H, which has been treated with silver nitrate. At the edges of the plate a randomized mixture of trimyristin and triolein was spotted as a reference standard. The diglyceride acetates applied to the interior of the plate show bands that correspond to the saturated, monounsaturated, diunsaturated, and triunsaturated glycerides in the reference sample. In addition, there is a fast-moving triene band, which can be seen just below the diene band. The tetraenes and pentaenes do not show up well in this photograph, but the major hexaene band can be seen at the origin. For an adequate quantitative examination, the polyene bands (tetraenes to hexaenes) were pooled from five to six such plates and were rechromatographed. Upon developing the plate two to three times in the same direction with 0.8% methanol in chloroform, it was possible to see and recover one tetraene band, a diffuse area containing various pentaenes, and two hexaene bands. Occasionally the diene band was separated into two components during the initial resolution; a major component was preceded by a minor one. The two diene bands showed the differences in the fatty acid composition, which are known to produce a resolution of diunsaturated triglycerides on silver nitrate plates (Table IV).

It should be noted that the intensity of

TABLE II
Major Diglyceride Acetates of Egg Yolk Lecithins[a]

Chemical classes	Carbon number[b]	Molecular species, mole % (1)		(2)		(3)		Average	
Saturates	32	6	1	4	1	3	2	4	1
	34	72		75		76		75	
	36	21		20		18		20	
	38	1		1		3		1	
Monoenes	34	2	49	3	47	1	50	2	49
	36	78		79		78		78	
	38	20		18		21		20	
Dienes I	34	2	4	0	5	1	3	1	4
	36	24		23		21		23	
	38	72		75		76		74	
	40	2		2		2		2	
Dienes II	36	64	31	66	32	69	35	66	33
	38	36		34		31		34	
Trienes I	36	16	3	24	2	20	1	20	2
	38	80		72		75		75	
	40	4		4		5		5	
Trienes II	36	5	1	1	1	0	2	3	1
	38	45		45		50		45	
	40	50		54		50		52	
Tetraenes	38	45	5	42	8	40	4	42	6
	40	54		56		57		56	
	42	1		2		3		2	
Pentaenes	36	10	Trace	8	Trace	8	Trace	9	Trace
	38	8		6		10		8	
	40	57		58		58		57	
	42	25		28		24		26	
Hexaenes I	38	10	5	15	3	20	3	15	4
	40	50		45		45		46	
	42	40		40		35		39	
Hexaenes II	40	30	Trace	20	1	28	Trace	25	Trace
	42	70		80		72		75	

Reconstituted mixture (average)	32	0.05	Original mixture (average)	32	Trace
	34	1.7		34	1.5
	36	62.5		36	63.4
	38	28.4		38	27.9
	40	5.8		40	6.0
	42	1.6		42	1.2

[a] Lecithins obtained from three different eggs from the same hen.
[b] The number of acyl carbon atoms in the molecule including that of the acetic acid.

fluorescence is not directly related to the concentration of the lipid bands on the thin-layer plates. Thus the diene band would appear to be the major band on the basis of the fluorescence shown in Fig. 1 when, in fact, it accounted for only about one-half of the material present in the monoene band, which showed much less fluorescence.

The diglyceride acetates, prepared from the lecithins isolated from the yolks of a dozen other eggs, showed nearly identical thin-layer chromatograms although small differences could usually be demonstrated in the gas chromatograms of the acetates and the fatty acids. Parallel analyses of samples from the same yolk showed excellent reproducibility.

Table II gives the molecular-weight distributions derived by direct gas chromatography of the original diglyceride acetates and of the diglyceride acetates recovered from the various bands of the silver nitrate plates. In the total acetate mixture, major peaks were seen for the diglyceride acetates containing one C_{16} and one C_{18} acid (C_{36}), and two C_{18} acids (C_{38}) per molecule. However small amounts of other molecular species were also present, as evidenced by the minor peaks for diglyceride acetates with carbon numbers of 34, 40, and 42. The carbon numbers of the diglyceride acetate peaks are derived by summing all the acyl carbons in the molecule, including the two carbons of the acetic acid residue.

The saturates were made up largely of molecules containing two C_{16} acids, and of molecules containing one C_{16} and one C_{18} acid. Traces were seen of diglyceride acetates with one C_{14} and one C_{16} acid as well as with two C_{18} acids. Other combinations of fatty acids giving total carbon numbers of 32 to 38 may also have been present, but, on the basis of the fatty acid proportions actually found in the fully saturated diglyceride acetate band, these could not have contributed significantly.

The monoenes contained major diglyceride

acetates with carbon numbers of 36 and 38, and traces of 34. The total diene fraction had a molecular-weight distribution which was nearly identical to that of the monoene fraction. Both the monoenes and the dienes were made up of C_{16} and C_{18} acids; most molecules contained at least one C_{18} acid. The diene band could usually be further resolved into two bands on the silver nitrate plate, in which case the faster-running minor band (Dienes I) showed the 36 and the 38 peaks in proportions opposite to those noted for the monoenes or the total dienes. As shown by determination of fatty acids (Table IV), the dienes with two monounsaturated fatty acids per molecule migrated faster than the dienes containing one diunsaturated and one saturated fatty acid in their molecules.

The trienes also showed two bands on silver nitrate-treated silica gel. The faster-moving fraction (Trienes I) contained a major peak for diglyceride acetates with a carbon number of 38, which consisted of one 18:1 and one 18:2 fatty acid per molecule. Smaller amounts of acetates with carbon numbers of 36, 40, and 42 could also be seen. The more slowly-moving triene band (Trienes II) mostly was made up of 16:0 and 18:0 acids in combination with 20:3 acid, but diglyceride acetates containing other triunsaturated acids were present. The tetraene band contained about equal proportions of diglyceride acetates with carbon numbers of 38 and 40. It was made up of diglyceride species with one 20:4 acid and one 16:0 or 18:0 acid residue per molecule.

Only a few satisfactory gas chromatograms were obtained with the pentaene bands, which showed rather complex distributions of molecular weights and apparently were made up of a variety of fatty acids combining to yield a total of five double bonds per molecule. Because of the small amounts of the material and its complex nature, the estimates presented for this fraction in Table IV are less precise than those for the other fractions.

The hexaenes were resolved into two bands under repeated development with the 0.8% methanol in chloroform. The more rapidly migrating band (Hexaenes I) contained one 22:6 acid per molecule in combination with 12:0 to 18:0 fatty acids. The other band (Hexaenes II) contained two unsaturated fatty acids per molecule; major contributions were made by linoleic and a 22:4 acid. This second hexaene band contributed only trace amounts of material to the total diglyceride acetate mixture.

The good agreement obtained between the proportions of the diglyceride acetates in the original mixture and those calculated from reconstitution shows that the method of analysis is quantitatively valid.

The fatty acid positions in the diglycerides were assigned on the basis of the data obtained from the pancreatic lipase hydrolyses of the total diglyceride acetate mixture and of the subfractions of the acetates recovered from the silver nitrate plates. In most cases it was sufficient to subject only the total—the monoene, diene, and the pooled polyene (trienes to hexaenes) fractions—to the lipase digestion. From the information acquired from the enzyme digestions and from the gas chromatography of the fatty acids and the diglyceride acetates, the composition of the original diglyceride mixture, hence of the original lecithin mixture, was reconstructed. After noting all the possible diglycerides that could be made

TABLE III
Reconstitution of the Composition of a Monoenoic Lecithin Fraction
(Yolk 1, Table III)

Experimental values						Calculated values[a]		
Glycerides	Mole %	Fatty acids	Mole %			Lecithins		Mole %
			Total	α	β	α	β	
C_{34}	2	16:0	40	80		16:0	16:1	2
		16:1	1.5		3	16:0	18:1	77
C_{36}	78	18:0	10	20		18:0	16:1	1
		18:1	48		96	18:0	18:1	19
C_{38}	20	20:1	0.5		1	16:0	20:1	1
						18:0	20:1	Trace

Calculations:

$(C_{34}) = (16{:}0\ 16{:}1) = 2\%$
$(C_{36}) = (16{:}0\ 18{:}1) + (18{:}0\ 16{:}1) = 78\%$
Since $(18{:}0\ 16{:}1) = 1\%$, $(16{:}0\ 18{:}1) = 77\%$
$(C_{38}) = (18{:}0\ 18{:}1) + (16{:}0\ 20{:}1) = 20\%$
Since $(16{:}0\ 20{:}1) = 1\%$, $(18{:}0\ 18{:}1) = 19\%$

[a] On a random basis it would have been anticipated that some 20:1 would also combine with 18:0; however the experimental values for the acids and the diglyceride acetates matched best when such a combination was excluded.

TABLE IV
Major Lecithins of Egg Yolks[a]

Chemical classes	Fatty acids[b] α	Fatty acids[b] β	Individual species, mole % (1)		(2)		(3)		Average	
Saturates	14:0	16:0	6		4		3		4	
	14:0	18:0	2		4		1		3	
	16:0	16:0	70	1	71	1	75	2	72	1
	18:0	16:0	21		20		18		20	
	18:0	18:0	1		1		3		1	
Monoenes	16:0	16:1	2		3		1		2	
	16:0	18:1	77		79		78		78	
	18:0	16:1	1	49	0	47	0	50	0	49
	18:0	18:1	19		18		20		19	
	16:0	20:1	1		0		1		1	
Dienes I	16:1	16:1	2		0		1		1	
	16:1	18:1	24		23		21		23	
	18:1	18:1	72	4	75	5	76	3	74	4
	18:1	20:1	2		2		2		2	
Dienes II	16:0	18:2	64		66		69		66	
	18:0	18:2	36	31	34	32	31	35	34	33
Trienes I	16:1	18:2	16		24		20		20	
	18:1	18:2	80	3	72	2	75	1	75	2
	20:1	18:2	4		4		5		5	
Trienes II	16:0	18:3	5		1		0		3	
	16:0	20:3	45		45		50		45	
	18:0	20:3	45	1	49	1	45	2	45	1
	16:0	22:3	5		5		5		7	
Tetraenes	16:0	20:4	45		42		40		42	
	18:0	20:4	54	5	56	8	57	4	56	6
	20:0	20:4	1		2		3		2	
Pentaenes	14:0	20:5	10		8		8		9	
	16:0	20:5	8		6		10		8	
	16:0	22:5	12	Trace	10	Trace	7	Trace	9	Trace
	18:1	20:4	45		48		51		48	
	18:1	22:4	25		28		24		26	
Hexaenes I	14:0	22:6	10		15		20		15	
	16:0	22:6	50	5	45	3	45	3	46	4
	18:0	22:6	40		40		35		39	
Hexaenes II	16:1	22:5	15		10		8		10	
	18:1	22:5	40		45		35		40	
	18:2	20:4	15	Trace	10	1	20	Trace	15	Trace
	18:2	22:4	30		35		37		35	

[a] Yolks obtained from three different eggs but the same hen.

[b] Fatty acid distribution established by specific lipase hydrolyses of the diglyceride acetates of the monoene, diene, and polyene bands.

from the fatty acids present, it was a matter of simple algebraic manipulation to obtain the proportions of the major diglyceride species in the mixture. Table III illustrates the method of calculation with data obtained for the monoene fraction of yolk 1, Table IV.

Table IV gives detailed accounts of the various molecular species of yolk lecithins of three eggs obtained from the same hen within one week. Apparently only small amounts of the fully saturated lecithins occur in this tissue. The major species are monounsaturated (16:0 18:1, 18:0 18:1) and diunsaturated (16:0 18:2, 18:0 18:2) lecithins; there are much smaller amounts of tetraunsaturated (16:0 20:4, 18:0, 20:4) and hexaunsaturated components (14:0, 16:0 or 18:0 with 22:6). For many of the tri- and penta- and some hexaunsaturated species, the identities and structure assignments must remain tentative. These latter lecithins however cannot account for more than a fraction of a per cent of the total mixture and cannot significantly influence the overall result. Even the latter could have been identified if larger amounts of material were worked up. In view of the need for a preliminary lipoprotein fractionation, however, it was felt that a more detailed study of these lecithins would not be worthwhile.

In this study more than 99% of the total egg yolk lecithin mixture has been accounted for in terms of molecules of exact composition, and their proportions in the total lecithin population have been measured. No comparable accounts of lecithin composition, of egg yolk or any other tissues, exist in the literature, and therefore no direct comparisons can be made. The present data are supported, however, by the results obtained from partial separations reported by Renkonen (2), in which 1-palmitoyl-2-monounsaturated acyl lecithins made up 74% and 1-stearoyl-2-monounsaturated acyl lecithins made up 26% of the monoene fraction. Furthermore the present estimates are in

excellent agreement with the proportions given by Renkonen for the diene fraction, which contained 1–palmitoyl–2–octadecadienoic acyl lecithins (64%) and 1–stearoyl–2–octadecadienoic acyl lecithins (36%) as major components. Also, according to Renkonen, the polyenes contained about equal mole proportions of saturated and polyunsaturated fatty acids. Similarly Lands and Hart (11) have shown that rat liver lecithins contain major amounts of mono-, di-, and tetraunsaturated species; the unsaturated components are in the β-position.

In contrast to the present data the lecithins examined by Renkonen (2) contained significantly more total monoenes (61.5–62.8%) and less dienes (22.0–23.6%) whereas the contributions of the saturates (2.1–3.3%) and polyenes (7.5–9.7%) were about the same as those found in the lecithin mixtures described in Table III. These differences in the proportions of the monoenoic and the dienoic lecithins are probably attributable to differences in the diets of the birds. Thus Rhodes (12) has reported, and Hanahan (1) has extensively discussed, experiments in which chicks were maintained on diets containing varying degrees of unsaturation in the component fatty acids. The lecithins of the eggs were isolated in the usual manner and assayed for unsaturation of the fatty acids in the α and β positions by means of lecithinase A. The predominating feature of the results was that, even though the proportion of the unsaturated fat in the diet was increased, only the β ester groups reflected the change. [Both Rhodes (12) and Hanahan (1) incorrectly assumed that lecithinase A was liberating unsaturated fatty acids from the α'– position (C–1) when, in fact, it was the β– or C–2 position that was attacked by this enzyme, as shown since by Tattrie (7)].

The existence of a metabolic heterogeneity in the lecithin fraction has been emphasized by Hanahan (1); Lands and Hart (11) have demonstrated that even individual component fatty acids must be regarded as having separate metabolic fates. Therefore, in order to determine the role of lecithin in membranes and lipoprotein enzymes, it will apparently be necessary not only to effect a complete fractionation of the lecithin species but also to establish that the isolated lecithins are representative of a specific subcellular fraction or lipoprotein. The yolk lecithins investigated in this study presumably came from the pool of lipoproteins present in the original egg yolk. The determination of the composition of the lecithins of specific yolk lipoproteins must await the development of methods for their isolation in pure condition.

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Ring Location in Cyclopropane Fatty Acid Esters by a Mass Spectrometric Method

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ABSTRACT

Ring location in cyclopropane fatty acid esters is accomplished simply and unequivocally with submilligram samples. The technique involves reductive ring opening with platinum catalyst and hydrogen in glacial acetic acid, to give a mixture of branched-chain and straight-chain acid esters. The sample is analyzed with a combination gas chromatograph-mass spectrometer. Examination of the spectra obtained from the mixture of branched-chain acid esters permits assignment of the position of the methyl groups, and hence of the ring in the parent compound.

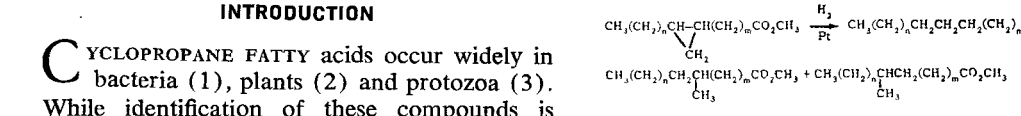
INTRODUCTION

CYCLOPROPANE FATTY acids occur widely in bacteria (1), plants (2) and protozoa (3). While identification of these compounds is relatively simple on a micro scale, using gas chromatography coupled with selective reduction (4), infrared spectrometry (4), and nuclear magnetic resonance (5), location of the position of the ring has been a difficult task, involving large samples of material. For example, the reductive ring opening procedure, followed by oxidative chain cleavage used by Kaneshiro and Marr (4) gives a very low yield of final product and requires several milligrams of starting material.

Complete characterization of naturally occurring cyclopropane acids, as well as biosynthetic studies on these compounds, requires a simple, rapid method of ring location which can be done with submilligram samples.

Although mass spectrometry has proven highly useful in the structure determination of fatty acids (6), one of its few failings in this respect has been the inability to locate cyclopropane rings or distinguish cyclopropane esters from their unsaturated isomers. It has been known for some time that both positional and geometrical isomers of isomeric monocyclopropane and monounsaturated esters give virtually indistinguishable mass spectra (7-9) which precludes the direct characterization of these compounds by mass spectrometry. Since the problem of double bond location has been solved for both long chain monoenoic esters (10) and hydrocarbons (11) through their O-isopropylidene derivatives, a satisfactory solution to cyclopropane ring location remained to be found.

A logical approach to the problem seemed to be the conversion of the cyclopropane moiety to a product or products bearing a close structural relationship to the original molecule, and which also exhibit characteristic mass spectra permitting assignment of ring position. These requirements are met by catalytic hydrogenation of the ring in the presence of Adam's catalyst and acetic acid which yields a straight chain ester and a mixture of the two possible branched chain isomers.



This reaction is easily carried out on a microgram scale, and the reaction mixture may be injected directly on to the gas chromatographic column connected to the mass spectrometer. The branched chain isomers precede and completely separate from the straight chain compound on both polar (e.g., DEGS) and nonpolar (e.g., SE-30) columns. No attempt is made to separate the branched compounds, which do not undergo any detectable separation under the conditions employed, as determined by repeatedly scanning mass spectra across the gas chromatographic peak as it is eluted from the column. Identification of peaks which are associated with the points of branching is then facilitated by their appearance in the spectrum 14 mass units apart, as will be discussed. Comparison of the mass spectrum of the mixture with those of the individual branched chain compounds (12) indicates that the branched isomers are formed in approximately equal proportion, and generally comprise about 80-90% of the products, the remainder being the straight chain isomer.

As we have recently demonstrated (13), cyclopropane esters corresponding to the more common unsaturated esters may easily be identified by comparison of mass spectra of the ring-opened products with reference spectra

such as Fig. 1-7. However, a detailed interpretation of the mass spectrum permits the unequivocal location of the cyclopropane ring without reference spectra, which may be highly useful in the characterization of new or unusual cyclopropane acids.

EXPERIMENTAL

Synthesis of Cyclopropane Esters

Fatty acids were obtained from commercial sources as follows: *cis*-9-tetradecenoic acid, *cis*-9-hexadecenoic acid, *cis*-9-octadecenoic acid and *cis*-5-eicosenoic acid, Applied Science Laboratories, State College, Pa.; *cis*-11-octadecenoic acid, and *cis*-6-octadecenoic acid, The Hormel Institute, Austin, Minn.

These acids were treated with diazomethane and the resulting methyl esters were converted to cyclopropane compounds by the method of Blanchard and Simmons (14). Samples of the esters (10-100 mg) were treated with excess diiodomethane and Zn-Cu couple in order to drive the reaction toward completion. The reaction mixtures were purified on $AgNO_3$-silicic acid columns (7) to yield the pure cyclopropane esters. The identity of these esters is based upon their gas chromatographic behavior (see 9) and upon their behavior on reduction.

TABLE I

Identity and Origins of Peaks in the Mass Spectra of Methyl Branched Fatty Acid Methyl Esters[a]

m/e	Designation in Fig. 1-7		Origin
74		$\left[CH_3OC(OH)=CH_2\right]^+$	Formed by rearrangement of hydrogen from C-4.
75		$CH_3OC(OH_2^+)=CH_2$	Formed by rearrangement of two hydrogens.[b] Origin of the second hydrogen is unknown.
		Types I, II and III: ions of the series 73 + n(14), where n = 1, 2, 3 . . .	
87, 101, 115, 129, 143, 157, 171, 185, etc.		I. $CH_3OCO(CH_2)_n^+$	Ions of this type are formed both by simple cleavage and by expulsion of groups from within the chain with hydrogen rearrangement.
	a	II. $CH_3OCO(CH_2)_n^+$	Same mass as I, but derived by bond breakage alpha to a methyl branch on the ester side. Neutral fragment lost is therefore a secondary radical.
	b	III. $CH_3OCO(CH_2)_nCH^+(CH_3)$	Same mass as I and II; alpha cleavage with formation of a secondary carbonium ion and loss of a primary radical.
144-145, 158-159, 172-173, 186-187, etc.	a + 1, a + 2		Rearrangement of one and two hydrogens from the remainder of the molecule to *a*.[b]
153, 156, 181, 195, etc.	*c*		Formed predominantly by elimination of CH_3OH from *b*.
135, 149, 163, 177, etc.	*d*		Due to elimination of H_2O from *c*.
M-76 (M = molecular ion)	*e*		Arises from methyl branch at C-6; probably involves elimination of CH_2OH and $CH_2{=}CHOH$. See ref. (16).
M-43			Loss of $-\overset{2}{C}H_2-\overset{3}{C}H_2-\overset{4}{C}H_2$ + H from within the chain.[c]
M-29			Loss of $-\overset{2}{C}H_2-\overset{3}{C}H_2-$ + H from within the chain.[c]
M-31			Loss of the methoxy group from the molecular ion (M).

[a] See Ref. 6. Contributions from isotope peaks are not listed (e.g., *m/e* 75, etc.).

[b] Also contains a small contribution from the isotope peak of the preceding peak.

[c] Shifts to a correspondingly lower mass if any of the atoms lost are substituted (17).

Dihydromalvalic acid (8, 9-methyleneheptadecanoic acid) was purified from the fatty acids of *Hibiscus syriacus* (following hydrogenation under mild conditions) and presented to us by Kim Hooper.

Reductive ring opening was accomplished by transferring a sample of cyclopropane ester (about 100 μg) to the bottom of a screw cap culture tube (1.5 cm $\times$ 12 cm), adding 2-3 mg PtO (Adam's catalyst) and 0.2 ml glacial acetic acid. The tube was placed inside the bottle of a Parr hydrogenation apparatus, and the sample was hydrogenated at room temperature and 20 psi, with shaking for 20 hr. The catalyst was filtered from the sample with the aid of a small amount of ethyl ether and the solvent was removed from the sample under reduced pressure. These reduced esters could be introduced into the gas chromatographic inlet of the mass spectrometer without further treatment.

Mass Spectra

Mass spectra were determined with an Atlas CH-4 mass spectrometer equipped with a gas chromatographic inlet system (1% SE-30, 100-120 mesh, in a 4-mm $\times$ 3-ft glass column, 140-185C). The transfer system including He separators was maintained at 180C and the ion source 250C. Ion source pressure was $\sim 2 \times 10^{-6}$ mm Hg; ionizing current 60 μamp; ionizing potential 20 ev. Uses of higher energies, e.g., 70 ev, made no significant difference in the relative intensities of the peaks in the spectrum. The required sample size depends on the quality of spectrum required, and ranges from approximately 0.1 to 3 μg. Comparison of standard and unknown spectra is possible with a spectrum of rather low intensity, while a search for metastable transitions in the spectrum of less common compounds necessitates greater absolute intensity. Experimental details of the combination instrument and discussion of the technique have recently been published (15).

RESULTS AND DISCUSSION

The mass spectra of branched chain esters are similar in many respects to those of straight chain esters (6). The presence of a methyl branch may frequently be recognized by the appearance of a peak of low relative intensity (usually less than 0.5%) at M-15, probably due to loss of the methyl branch. Origins of the most common peaks in these spectra are summarized in Table I.

Due to the great relative intensity of m/e 74,

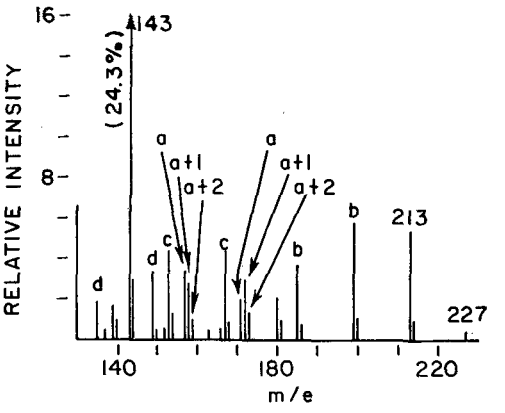


FIG. 1. Partial mass spectrum of the branched acid esters derived from methyl 9,10-methyleneoctadecanoate (methyl dihydrosterculate).

the most intense peak in every case studied, many of the peaks of diagnostic interest are of low relative intensity when normalized to m/e 74. However, the wide dynamic range (about 5000) inherent in multitrace recording and in the technique itself still results in a highly accurate and reproducible recording of the less abundant ions even with very small samples. Therefore, for more legible representation, Fig. 1-5 are shown as enlarged partial spectra[1] of the mass region of interest, and contain all peaks of relative intensity greater than 0.5%.

Of primary interest for location of the cyclopropane ring (via the branch locations) are ions *a*, *a* + *1*, and *a* + *2* of the 73 + n(14)

[1]Complete mass spectra in tabular form of Fig. 1-7 are available on request.

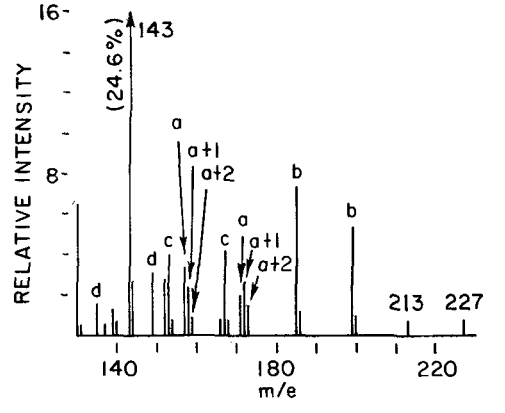


FIG. 2. Partial mass spectrum of the branched acid esters derived from methyl 9,10-methyleneenehexadecanoate.

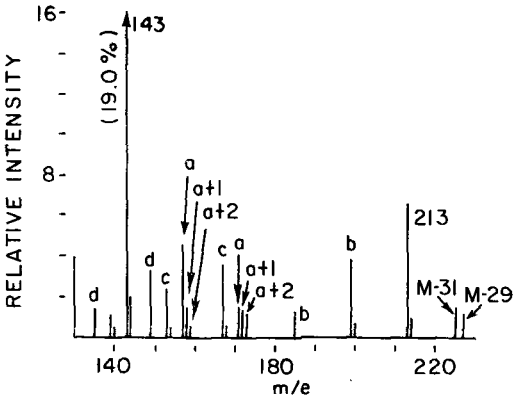
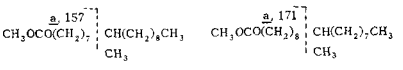


FIG. 3. Partial mass spectrum of the branched acid esters derived from methyl 9, 10-methylenetetradecanoate.

series. Although three different types of ions (I, II, III in Table I) contribute to the 73 + n(14) series, only the primary ion *a* formed by alpha cleavage to the points of branching will undergo abstraction of one and two hydrogens to yield *a* + *1* and *a* + *2* (6). The resulting characteristic sets of three adjacent peaks 14 mass units apart are easily recognized and used to establish the points of branching, as shown by comparison of Fig. 1 and 4. Each component of the mixture thus gives rise to a set of *a* peaks, which as for the esters derived from methyl dihydrosterculate (Fig. 1), occur 14 mass units apart.



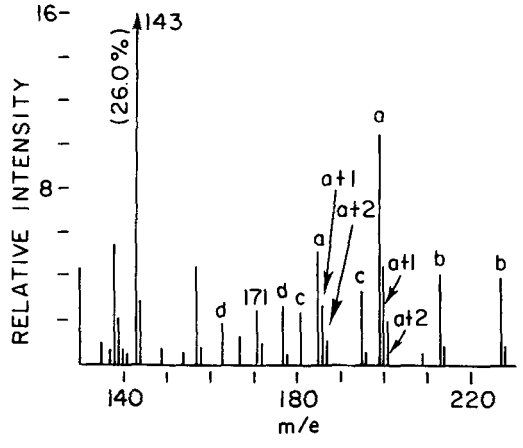


FIG. 4. Partial mass spectrum of the branched acid esters derived from methyl 11, 12-methyleneoctadecanoate (methyl lactobacillate).

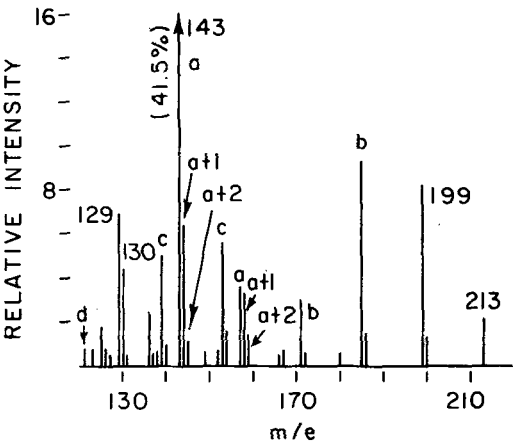


FIG. 5. Partial mass spectrum of the branched acid esters derived from methyl 8, 9-methyleneheptadecanoate.

For closely related compounds differing only in chain length, as in Fig. 1 and 2, the ratios of the three ions will be very similar. The intensities of *a* + *1* and *a* + *2* relative to *a* may be very low in cases in which ions of type I (Table I) contribute strongly to the mass value at which *a* also falls. The most extreme example would occur with branched esters derived from 7, 8 or 8, 9-cyclopropane esters. In this latter case (shown in Fig. 5) the branch at C-8 gives rise to an ion of the type $CH_3OCO(CH_2)_6^+$ (*m/e* 143), which is generally much more intense in long chain ester spectra (6) than other members of the 73 + n(14) series with the exception of *m/e* 87 n=1. However, *a* + *1* and *a* + *2* are still readily recognized at *m/e* 144 and 145. Mass 144 in Fig. 5 is clearly in excess of the usual intensity of that peak (see Fig. 1-4), due mainly to the C^{13} isotope peak of *m/e* 143.

The greater stability of secondary over primary carbonium ions gives rise to cleavage at points of branching, yielding ions of type *b* (Table I). Mass spectral peaks representing such preferential cleavage should therefore in

TABLE II.

Metastable Peaks from the Mass Spectrum of the Branched Acid Esters Derived from Methyl 9, 10-Methyleneoctadecanoate (Fig. 1)

m/e Calcd.	*m/e* Found	Transition indicated, *m/e*
140.1	140.2	199 (*b*) → 167 (*c*)
132.9	133.0	167 (*c*) → 149 (*d*)
126.5	126.6	185 (*b*) → 153 (*c*)
119.1	119.1	153 (*c*) → 135 (*d*)

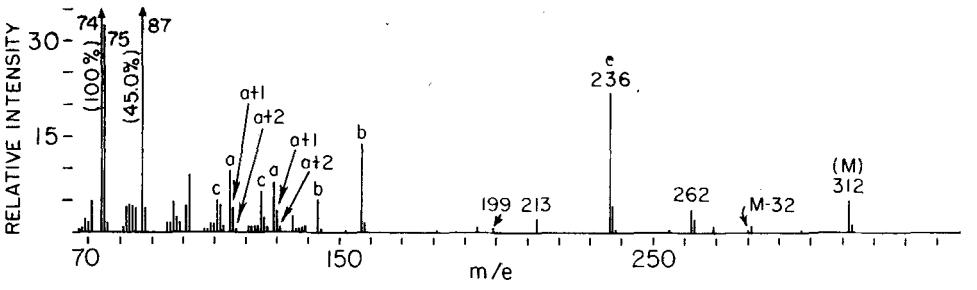


FIG. 6. Mass spectrum of the branched acid esters derived from methyl 6, 7-methyleneoctadecanoate.

principle be useful for establishing the position of the branch; however, ions of type I (but not type II) may also fall at the same mass as *b* in any given spectrum, making the recognition of *b*, unlike *a* + *1* and *a* + *2*, a matter of intensity. Therefore *b* may generally be used to corroborate the points of branching as determined by the *a* series. Thus *m/e* 227 (*b*) in Fig. 4 is more intense than *m/e* 227 in Fig. 1 or 2, where it is an ion of type I. Each *b* ion must be found 28 mass units higher than its corresponding *a*, since the structural difference is $-\dot{C}-CH_3$. For instance, although *m/e* 213 in Fig. 1 (due mainly to the 9-methyl component (12)) appears to be unusually intense for type I (compare with *m/e* 213 of Fig. 2) it does not appear 28 mass units higher than either of ions *a*, as required for *b*. In order to make approximate intensity predictions in the spectra of the mixed branched chain esters, reference may be made to the extensive compilation of 70 ev spectra of individual branched esters published by Ryhage and Stenhagen (12).

Elimination of methanol from *b* results in ions of type *c* (e.g., *m/e* 199→*m/e* 167 in Fig. 1). This process has generally been postulated to be a 1, 2-elimination, yielding ketene-type ions (12,6), although it has been suggested (16) that this earlier assumption was incorrect. Further loss of water from *c* gives ion *d* (see Fig. 1-5), a process which on energetic grounds would seem less likely from a ketene structure. On the other hand, elimination of water from cyclic ketones, a likely product from a 1,n elimination of methanol (where n > 2), is a minor but commonly observed process (17). The sequence *b*→*c*→*d* (12) is substantiated by appropriate metastable transitions, listed in Table II. Although ions which are 32 and (32 + 18) mass units lower than type I ions may be observed in some cases (e.g., *m/e* 181 and 163 in Fig. 1), their intensities are considerably less (*m/e* 167, 149, Fig. 1) than analogous ions derived from *b*. Though the apparent absence of metastable transitions does not disprove the relationship between ions (i.e., that *m/e* 181 is, or is not derived by loss of methanol from *m/e* 213), the observation of reasonably intense metastable transitions may be used to corroborate the identity of *b*, *c* and *d* ions.

Cyclopropane esters containing the methylene bridge at C-5, 6 or 6, 7 yield branched chain esters which exhibit mass spectra with a considerably different appearance, as shown

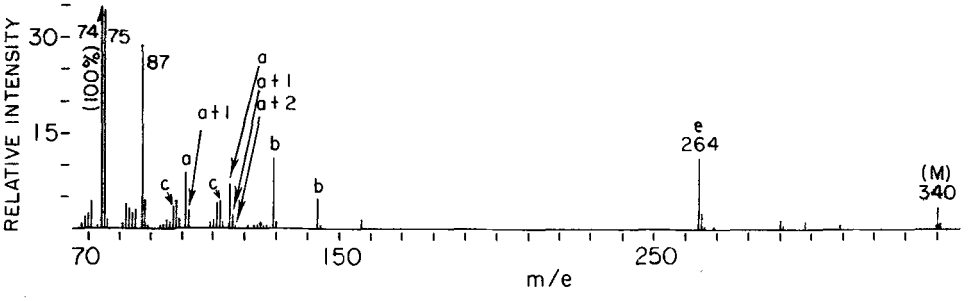
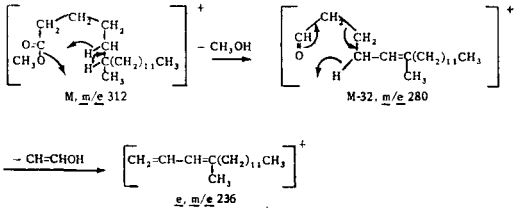


FIG. 7. Mass spectrum of the branched acid esters derived from methyl 5, 6-methyleneeicosanoate.

by comparison of Fig. 1-5 with spectra derived from methyl 6,7-methyleneoctadecanoate (Fig. 6) and methyl 5, 6-methyleneeicosanoate (Fig. 7). The spectra represented in Fig. 6 and 7 are dominated in the upper mass range by M-76, ion *e*. Although M-76 is observed at very low abundance in spectra of 5- and 7-methyl branched esters (12), its presence as an intense peak is characteristic of a methyl branch at C-6 (12), and may thus be used to establish the location of the original cyclopropane ring as either 5, 6 or 6, 7. Ions of type *a* are then restricted to values of *m/e* 101 and 115 (5, 6 position, Fig. 7) or 115 and 129 (6, 7 position, Fig. 6) and are easily recognized, even though *b* and *c* are less obvious (or may be doublets) and *d* may be absent. In analogy to the origin of the M-76 ion in mass spectra of methyl 6-phenylhexanoate, Meyerson and Leitch have suggested that the same mechanism operates in the case of the 6-methyl branched esters (18). In terms of the 6-methyl component of the branched chain mixture derived from methyl 6,7-methyleneoctadecanoate (Fig. 6) the mechanism may then be formulated as:



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Hepatic Lipid Metabolism in Experimental Diabetes: III. Synthesis and Utilization of Triglycerides

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ABSTRACT

Livers removed from normal rats, from alloxan diabetic rats maintained on insulin for two weeks (ADI+), and from insulin-treated diabetic rats from which insulin had been withdrawn two days before use (AD) were perfused in vitro with 120 mg (468 μmoles) palmitic acid-1-C^{14}. Under these conditions, output of TG (triglyceride) was depressed in livers from ADI+ rats and was negligible with livers from AD animals. The total incorporation of C^{14} into perfusate TG paralleled the chemical measurements of TG output. The concentration of hepatic TG increased during perfusion of livers from normal or ADI+ rats but decreased during perfusion of livers from AD animals.

A load of 120 mg of palmitic acid/3 hr was inadequate to maintain net accumulation of TG in livers from AD rats; furthermore it is implicit in this observation that the total load of NEFA (nonesterified fatty acid) perfusing livers from AD rats must be increased considerably to obtain a fatty liver. The total incorporation of C^{14} into hepatic TG and the specific activity of hepatic TG were depressed during perfusion of livers from AD rats. The production of ketone bodies by livers from AD animals was about five times the normal rates; the output of ketone bodies did not differ from results of other experiments (1) in which the load of palmitic acid added to the medium was varied from 0–80 mg. These observations were discussed with reference to mechanisms for ketogenesis and fatty liver in alloxan diabetes.

INTRODUCTION

IT HAS BEEN REPORTED PREVIOUSLY that the isolated perfused rat liver released triglyceride (TG) into the medium in direct proportion to the load of fatty acid which was presented to it (1). Regardless of the load of nonesterified fatty acid (NEFA), the rate of release of triglyceride by livers from acute alloxan diabetic rats (prepared 48 hours before use) was always less than that observed with livers from normal rats. The release of triglyceride into the very-low-density lipoprotein of the perfusate, the total incorporation of palmitate-1-C^{14} into triglyceride, and the specific activity of the triglyceride released into this lipoprotein fraction were depressed during perfusion of livers from diabetic rats (2). In the intact rat made diabetic with alloxan, the concentration of triglyceride in the liver is increased considerably above normal levels; when the livers from acute alloxan diabetic rats were perfused in vitro with a medium containing a relatively small load of palmitic acid, the concentration of hepatic triglyceride fell during perfusion to levels which were not different from those seen in perfused livers from normal animals (2).

The impairment in release of triglyceride by livers from alloxan diabetic rats may, in part, result from an increased rate of lipolysis of hepatic triglyceride. If this were so, the load of nonesterified fatty acid employed in previous experiments might have been insufficient to maintain net synthesis of triglyceride in the face of greatly increased rates of lipolysis of triglyceride. The effect of increased palmitate-1-C^{14} load on the transport and metabolism of triglyceride by livers from normal, diabetic, and controlled diabetic rats has been investigated further in the work to be reported in this paper. "Chronic" diabetic rats were employed in the present studies to circumvent any acute toxic effect of alloxan on the liver.

METHODS

Livers obtained from normal rats, diabetic rats, and diabetic rats treated with insulin were perfused in vitro by using the general procedures (3) and apparatus (4) which have been described in detail in earlier publications.

Normal male rats (obtained from Holtzman Company, Madison, Wis.), weighing 350–450 g, were maintained on a balanced food ration and served as sources of blood and serum for all

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experiments. The animals from which the livers were obtained were divided into three groups. One group consisted of normal male rats (body weights ranged from 370 to 420 g). A second group consisted of male rats made diabetic by the intravenous injection of alloxan monohydrate (60 mg/kg body weight); 48 hr later, diabetes was confirmed by measurement of the elevation of blood glucose. The rats were then given subcutaneous injections daily of Protamine Zinc Insulin (12 I.U./kg of body weight) for two weeks; these animals received the last injection of insulin approximately two to three hours before the livers were removed for perfusion. During this period the rats gained weight, and the urine was free of glucose and ketone bodies (estimated qualitatively with Clinistix and Ketostix, Ames Company, Elkhart, Ind.). This group is referred to as controlled chronic diabetic rats. The blood glucose of animals in this group varied from 35 to 70 mg % at the time of surgical hepatectomy. A third group consisted of controlled chronic diabetic rats, from which insulin had been withdrawn 48 hr before use. This latter group is referred to as chronic diabetic rats. During this period of two days the rats lost weight and became glucosuric and ketonuric. The blood glucose concentration of these animals, determined according to the procedure of Nelson (5), was in excess of 350 mg % at the time of the liver removal. Animals in all groups were allowed free access to food (Purina Laboratory Chow) and water.

Immediately following its surgical removal from the animal, the liver was inserted into the perfusion apparatus; it was then perfused for 20 min with a medium consisting of 50 ml of defibrinated rat blood, 500 I.U. of heparin, and sufficient Krebs-Henseleit bicarbonate buffer, pH 7.4 (6), to make a volume of 70 ml. After the equilibration period 30 ml of a complex of rat serum and palmitate-1-C^{14} were added to the perfusion medium. The serum complex consisted of 20 ml of rat serum, 10 ml of 0.9% NaCl, and 40 mg (156 μmoles) of palmitic acid-1-C^{14} (specific activity = 12.82 mμC/μmole palmitic acid) (7). Ten ml of perfusate were removed for analysis 4 min after the palmitate-serum complex was added to the medium. Immediately thereafter an infusion of palmitic acid-rat serum complex was started and was continued until an additional 312 μmoles of palmitic acid-1-C^{14} (80 mg) had been infused. The infusion rate was 0.37 ml/min or 2.3 μmoles/min. The composition of the infused palmitic acid-serum complex was as follows: 40 ml of rat serum, 10 ml of 0.9% NaCl, and 312 μmoles (80 mg) of palmitic acid-1-C^{14}. Additional 10-ml aliquots of perfusate were removed for analysis 90 and 180 min after the first sample had been taken. At the termination of the experiment the livers were removed from the apparatus, were perfused with ice-cold 0.9% NaCl solution to remove blood, were trimmed free of extra hepatic tissue; blotted dry and weighed; then were assayed for lipid content. The hepatic content of the lipid of perfused livers was compared with livers which had not been perfused. For this purpose, rats from the three experimental groups were anesthetized and their livers removed as for perfusion; the livers were not inserted into the perfusion apparatus however, but were assayed immediately for content of lipid.

The lipids of the livers (8) and of samples of perfusate which had been centrifuged to remove erythrocytes (1) were extracted as described earlier. Aliquots of the extracts were evaporated to dryness in vacuum at 50C, were dissolved in a minimal volume of $CHCl_3$, and were placed on 3.0-g silicic acid columns (1). Aliquots of the $CHCl_3$ eluates (35 ml) of the silicic acid columns were evaporated to dryness, taken up in a minimal volume of $CHCl_3$, and banded on thin-layer silicic acid chromatography plates. Triglycerides and NEFA were separated from other lipids (9) and eluted from the plates with $CHCl_3$; aliquots of the eluates were taken for chemical and isotopic analysis. Triglycerides were estimated by the method of Van Handel and Zilversmit (10). Nonesterified fatty acids were determined according to the procedure of Duncombe (11). For the measurement of hepatic phospholipid, aliquots of CH_3OH eluates (35 ml) of the silicic acid columns were taken to dryness, were dissolved in a minimal volume of $CHCl_3$, and were chromatographed on thin-layer silicic acid plates to remove contaminating trace amounts of C^{14}-fatty acids (9). The band of PL (phospholipid) was eluted from the plates with a mixture of ethanol: chloroform: distilled water: glacial acetic acid (100:30:20:2, v/v) (12). The eluates were evaporated to dryness in vacuum and redissolved in $CHCl_3$; aliquots of the eluates were taken for analysis. Lipid-soluble phosphorous was measured by the method of King (13).

The radioactivity present in TG, NEFA, and PL was measured on samples of the appropriate eluates from the silicic acid plates. The solvent was evaporated in vacuum; the lipids

TABLE I
Concentration and Specific Activity of Nonesterified Fatty Acid in the Perfusion Fluid[a]

Group	Concentration[b] μeq/ml cell free perfusate Minutes 0	90	180	Specific activity[c] (DPM/μeq) x 10^{-4} Minutes 0	90	180
Normal (5)	1.47±0.05	0.44±0.04	0.19±0.05	2.41±0.07	1.43±0.18	0.80±0.21
Controlled diabetic (5)	1.26±0.18	0.37±0.02	0.14±0.02	2.73±0.33	1.76±0.10	1.16±0.30
Diabetic (6)	1.15±0.16	0.34±0.02	0.14±0.03	2.97±0.11	1.77±0.10	1.02±0.18

[a] All values are means ± standard error. The decline of the concentration and specific activity of NEFA were not statistically different among groups. Figures in parentheses indicate number of experiments.

[b] Assuming instantaneous mixing of added NEFA, the theoretical initial concentration of NEFA (4 minutes before zero time) was 2.08 μeq/ml cell free supernatant.

[c] Specific activity of added NEFA = 2.94 x 10^4 DPM/μeq.

were dissolved in toluene containing 4 g of diphenyloxazole and 100 mg of p-bis-1, 2 (phenyloxazole)-1-benzene per liter and were counted in a Tracerlab liquid scintillation counter (Model LSC-10B).

One-ml aliquots of samples of perfusate, which were obtained at each period, were added to 3 ml of distilled water and were allowed to hemolyze. One ml each of 0.15 N Ba $(OH)_2$ and 2.5% $ZnSO_4 \cdot 7H_2O$ was added to the hemolysate to precipitate the proteins. Suitable samples of protein-free filtrate were analyzed for ketones by the method of Michaels (14) after oxidation in the apparatus described by Greenberg and Lester (15).

The statistical significance of the differences between groups and treatments was evaluated with Student's distribution for "t" (two-tailed table) (16). The data presented in all tables and figures were derived from the same perfusion experiments during which the various parameters were measured.

RESULTS

Disappearance of Palmitate-1-C^{14} from the Perfusate

Table I presents the concentrations and specific activities of nonesterified fatty acid in the perfusate. The concentration of NEFA in the medium declined rapidly during perfusion of livers from all groups of animals despite the infusion of palmitate-1-C^{14} during the course of the experiment. This rapid rate of removal of palmitate from the medium by the liver agrees with the data obtained by several investigators (17–19). There were no significant differences in the concentration of NEFA in the perfusate among groups at any time. The specific activities of NEFA declined during the perfusions; there were however no differences in the specific activities of NEFA among groups.

Net Release of Triglyceride by Perfused Livers

The rate of accumulation of TG in the medium when livers from normal, chronic diabetic, and controlled chronic diabetic rats were perfused is shown in Figure 1. An increase in the concentration of TG in the medium was observed with all preparations. The increase in concentration of TG was relatively linear during the 180-minute perfusion of livers from either normal or controlled diabetic rats. The rate of accumulation of TG in the medium, when livers from controlled diabetic rats were perfused, approached that observed when livers from normal rats were perfused.

In contrast to these groups, the rate of accumulation of TG in the perfusate was impaired when livers from diabetic rats were

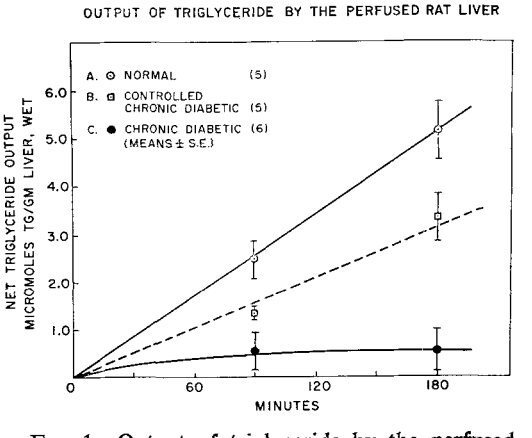

FIG. 1. Output of triglyceride by the perfused rat liver. Vertical bars in figure denote ± one standard error.

Probability:

	0-90 min.	0-180 min.
A *vs.* B	< 0.02	< 0.06
A *vs.* C	< 0.01	< 0.001
B *vs.* C	< 0.10	< 0.005

TABLE II

Incorporation of Palmitate-1-C^{14} into Triglyceride of the Perfusion Fluid[a]

Group	Total incorporation (DPM/g liver)[b] x 10^{-4} Minutes 0	90	180	Specific activity (DPM/μmole TG) x 10^{-4} Minutes 0	90	180
A. Normal (5)	0.10±0.02	11.82±1.34	23.19±2.79	0.05±0.01	2.66±0.23	3.28±0.13
B. Controlled diabetic (5)	0.04±0.01	8.29±0.60	14.30±1.40	0.03±0.01	3.32±0.24	3.34±0.27
C. Diabetic (6)	0.04±0.01	2.09±0.35	2.75±0.63	0.02±0.004	1.02±0.22	1.28±0.22
Probability:						
A *vs.* B	.....	<0.05	<0.025	.....	N.S.	N.S.
A *vs.* C	.....	<0.001	<0.001	.....	<0.001	<0.001
B *vs.* C	.....	<0.001	<0.001	.....	<0.001	<0.001

[a] All values are means ± standard error. Figures in parentheses indicate number of experiments.
[b] Wet weight.

perfused. In fact, no net accumulation of TG was noted between 90 and 180 min. It was reported previously that livers from normal and acute alloxan diabetic rats released TG into the perfusate in proportion to the total load of palmitate presented to the livers (1). A loss of TG from the perfusate was observed when livers from acute diabetic rats were perfused with loads of palmitic acid less than 312 μmoles; this was also observed when livers from chronic alloxan diabetic rats were perfused. In these experiments a load of 468 μmoles of palmitate was provided the liver during the period of perfusion.

Incorporation of Palmitate-1-C^{14} into Triglyceride of the Perfusate

The total incorporation of palmitate-1-C^{14} and the specific activity of the triglyceride of the perfusate can be seen in Table II. The incorporation of palmitate-1-C^{14} into TG paralleled the rates of accumulation of TG in the perfusate. The incorporation of palmitate-1-C^{14} into TG of the perfusate was less than normal when livers from controlled chronic diabetic rats were perfused and was a minimum when livers from chronic diabetic rats were perfused. No net increase in the incorporation of palmitate-1-C^{14} into TG was observed between the 90-minute and 180-minute period when livers from chronic diabetic rats were perfused.

The specific activity of the perfusate TG reached a maximum after 90 min of perfusion and was the same with preparations from either normal or controlled diabetic rats. The specific activity of perfusate TG when livers from diabetic rats were perfused was about 0.3 that obtained when the livers from normal or controlled diabetic rats were perfused.

Hepatic Triglyceride Concentration

The data presented in Table III are the mean values for the concentration of hepatic TG in livers from normal, controlled diabetic, and diabetic rats. When the livers were not perfused, the concentration of TG in the organs from diabetic rats was observed to be three to four times greater than that which was estimated in livers from normal or controlled diabetic rats. After 3 hr of perfusion the livers from animals in each of the experimental groups had the same TG concentration. The concentration of TG in perfused livers from normal and from controlled diabetic rats was significantly higher than that in the corresponding livers which had not been perfused. The mean concentration of TG in perfused

TABLE III

Hepatic Concentration of Triglyceride[a]

Group	μmoles TG/g liver, wet weight Not perfused[b]	Perfused[c]	Probability[d]
A. Normal	8.29±1.20 (7)	13.30±0.45 (5)	<0.01
B. Controlled diabetic	6.39±1.14 (6)	11.78±0.97 (5)	<0.01
C. Diabetic	26.46±8.28 (6)	13.90±2.02 (6)	<0.20
Probability:			
A *vs.* B	N.S.	N.S.	
A *vs.* C	<0.05	N.S.	
B *vs.* C	<0.05	N.S.	

[a] All values are means ± standard error. Figures in parentheses indicate number of experiments.
[b] The concentration of TG in the perfused livers at the beginning of the experiment was considered statistically to be identical with the concentration of TG in livers which were not perfused.
[c] The concentration of TG in perfused livers is that measured at the termination of the experiment (3 hr).
[d] Significance of differences between perfused livers and livers which have not been perfused.

TABLE IV

Incorporation of Palmitate-1-C^{14} into Hepatic Triglyceride[a]

Group	Total incorporation (DPM/g liver)[b] x 10^{-4}	Specific activity (DPM/μmole TG) x 10^{-4}
A. Normal (5)	28.68±2.92	2.16±0.23
B. Controlled diabetic (5)	27.52±1.14	2.38±0.16
C. Diabetic (6)	11.33±2.03	0.81±0.09
Probability:		
A *vs.* B	N.S.	N.S.
A *vs.* C	<0.001	<0.001
B *vs.* C	<0.001	<0.001

[a] All values are means ± standard error. Figures in parentheses indicate number of experiments. All analytical measurements were made at the termination of the perfusion period (3 hr).

[b] Wet weight.

TABLE VI

Incorporation of Palmitate-1-C^{14} into Hepatic Phospholipid[a]

Group	Total incorporation (DPM/g liver)[b] x 10^{-4}	Specific activity (DPM/μmole) x 10^{-2}
A. Normal (5)	14.91±0.50	38.35±0.77
B. Controlled diabetic (5)	13.70±0.48	37.84±1.96
C. Diabetic (6)	8.29±0.79	27.74±2.24
Probability:		
A *vs.* B	N.S.	N.S.
A *vs.* C	<0.001	<0.001
B *vs.* C	<0.001	<0.005

[a] All values are means ± standard error. Figures in parentheses indicate number of experiments. All analytical measurements were made at the termination of the perfusion period (3 hr).

[b] Wet weight.

livers from diabetic rats was approximately 0.5 that in livers which had not been perfused; this difference in the means however was not statistically significant. The conclusion that can be derived from these data is that livers from normal and controlled diabetic rats accumulated TG during perfusion whereas livers from diabetic rats lost, or did not gain, TG when the livers were exposed to a load of 468 μmoles of palmitic acid.

Incorporation of Palmitate-1-C^{14} into Hepatic Triglyceride

It can be seen that livers from normal and controlled diabetic rats incorporated the same amount of palmitate-1-C^{14} into hepatic triglyceride (Table IV). Livers from diabetic rats incorporated approximately 0.3 as much radioactivity into hepatic TG as was incorporated by livers from normal and controlled diabetic animals. Similarly the specific activity of TG in perfused livers from diabetic rats was about 0.3 the specific activity of TG in livers obtained from normal or controlled diabetic rats.

TABLE V

Hepatic Concentration of Phospholipid[a]

Group	μmoles PL/g liver, wet weight: Not perfused	Perfused	Probability[b]
A. Normal	43.05±1.00 (8)	38.93±1.27 (5)	<0.05
B. Controlled diabetic	43.40±3.09 (7)	36.40±1.71 (5)	<0.15
C. Diabetic	42.31±3.37 (7)	33.47±1.82 (6)	<0.05
Probability:			
A *vs.* B	N.S.	N.S.	
A *vs.* C	N.S.	<0.02	
B *vs.* C	N.S.	N.S.	

[a] All values are means ± standard error. Figures in parentheses indicate number of experiments. The concentration of PL in perfused livers is that measured at the termination of the experiment (3 hr).

[b] Significance of differences between perfused livers and livers which have not been perfused.

Hepatic Phospholipid Concentration and Incorporation of Palmitate-1-C^{14}

The concentration of phospholipid in livers which were not perfused and in perfused livers from normal, controlled diabetic, and diabetic rats is presented in Table V. The concentration of PL in livers which were not perfused did not differ among groups. Perfused livers from diabetic rats however contained less PL than perfused livers from normal rats. In general, it appeared that the concentration of PL in the liver declined in all groups during perfusion.

It can be seen in Table VI that livers from diabetic rats incorporated palmitate-1-C^{14} into hepatic PL to a lesser degree than did livers from normal or controlled diabetic rats. The specific activity of PL in livers from diabetic rats was also depressed in comparison to the control groups.

TABLE VII

Production of Ketone Bodies by the Perfused Rat Liver[a]

Group	mg Acetone/g. liver, wet weight: Minutes of perfusion 90	180
A. Normal (5)	0.82±0.12	1.43±0.26
B. Controlled diabetic (5)	0.63±0.06	1.09±0.12
C. Diabetic (6)	3.47±0.65	4.94±0.76
Probability:		
A *vs.* B	.....	N.S.
A *vs.* C	.....	<0.005
B *vs.* C	.....	<0.005

[a] All values are means ± standard error. Figures in parentheses indicate number of experiments.

Ketone Body Production

The output of ketone bodies by livers from diabetic rats was about five times greater than that by livers from normal rats (Table VII). Livers from controlled diabetic rats produced ketone bodies at a rate similar to that of livers from normal rats.

DISCUSSION

The release of triglyceride by livers from diabetic rats is depressed severely in contrast to the normal. It is unlikely that these observations resulted from variations in substrate (NEFA) concentration or from differences in precursor specific activity. In addition to a lack of differences among experimental groups in the concentration or the specific activity of NEFA in the medium, the concentration of NEFA in the livers from controlled diabetic rats and from diabetic animals was identical at the end of perfusion (0.72 ± 0.20 and 1.07 ± 0.15 μmoles/g liver, wet weight, respectively; $P < 0.2$), as was also the specific activity (4210 ± 322 and 3844 ± 835 dpm/μmole, respectively; $P < 0.8$). The decrease in specific activity of the NEFA of the medium during the experiment may have been a consequence of dilution by NEFA of lower specific activity; whether the additional NEFA arose from the liver or resulted from hydrolysis of triglyceride in the medium cannot be determined from these experiments. The concentration of TG in the medium however and the C^{14} present in the TG remained constant when the medium was perfused in the apparatus in the absence of the liver (M. Heimberg and I. Weinstein, unpublished experiments).

It is most probable that the biosynthesis of phospholipid by the livers from the chronic alloxan diabetic rats was depressed in comparison with the normal, in agreement with observations made in experiments in which livers from acute alloxan diabetic animals were perfused (2). The hepatic triglyceride which was being degraded during the course of the perfusion may have contributed to the pool of fatty acid, diluted the specific activity of the precursor palmitic acid C^{14} with nonisotopic fatty acid, and resulted, in part, in an apparent inhibition of phospholipid biosynthesis. It is not possible to conclude from the data with any reasonable degree of certainty that the esterification of free fatty acids to triglyceride was also reduced in livers from chronic alloxan diabetic rats; unlike the phospholipids, wherein there was identity of magnitude of hepatic pools in all experimental groups, the concentration of triglyceride in livers from chronic alloxan diabetic animals at the beginning of the experiments was greater than that in the livers from control animals. It can be seen that, were equal numbers of molecules of palmitic acid-1-C^{14} converted into liver triglyceride by all groups, the specific activity would be reduced in the diabetic to about 0.3 that of the control groups. Furthermore, were the triglyceride pool oxidized more rapidly in the livers from the diabetic than from the control animals, without any further dilution of triglyceride specific activity, data similar to those obtained might be expected. To resolve the question of inhibition of hepatic biosynthesis of triglyceride as a result of alloxan diabetes, it might be more appropriate to employ enzyme preparations, in which substrate and enzyme concentration can be regulated, or to maintain the perfused liver under steady state conditions, wherein both perfusate concentration of NEFA and hepatic concentration of TG remain constant.

Some interesting comparisons of TG metabolism can be made between livers from normal rats and those from chronic alloxan diabetic rats which were controlled with insulin. The TG that was released by the liver in either of these two experimental groups must have arisen from identical metabolic pools since the hepatic concentration of TG and the specific activity of hepatic and perfusate TG were the same in both groups. It can be surmised from these data and from the apparent inhibition of net release of TG that a partial block in outward transport of TG existed in the livers from the chronic alloxan diabetic rats even though the animals were maintained on insulin. The control of diabetes with single daily injections of protamine zinc insulin was evaluated in these experiments by the usual criteria of absence of glucosuria, ketonuria, and hyperglycemia, also by the presence of weight gain; these treatments however may not have been adequate to assure a normal hepatic lipid metabolic pattern.

Data also are suggestive of a block in the outward transport of TG in livers from diabetic animals, in addition to any inhibition of the biosynthesis of triglyceride or acceleration of lipolysis of TG. This conclusion is derived from the following observations. The total incorporation of palmitic acid-1-C^{14} into hepatic TG by livers from chronic alloxan diabetic animals and the specific activity of the hepatic

TG were about 0.3 that of the controls. Although the specific activity of the TG which was released by the livers from diabetic animals into the perfusate was also about 0.3 of that released by livers from normal rats, the total incorporation at the end of three hours of perfusions was only about 0.1 that of the normal. It was reported previously that chylomicron triglyceride was removed from the perfusate by livers from alloxan diabetic rats at a faster rate than by livers from normal animals or from diabetic rats treated with insulin (1); thus the impairment in net release of triglyceride may result, in part, from an increased rate of re-uptake of perfusate triglyceride and, in part, from an inhibition of the rate or release of the very-low-density lipoprotein triglyceride by the livers from diabetic animals.

In these experiments the livers from normal and from controlled diabetic rats accumulated TG during the perfusion whereas livers from diabetic rats did not. In similar experiments, in which only a 40-mg load of palmitic acid was used, livers from normal rats showed no change in hepatic triglyceride concentration during perfusion whereas livers from acute alloxan diabetic rats lost significant quantities of triglyceride (2). The accumulation of TG in the perfused liver in vitro (D. R. Van Harken, C. Dixon, and M. Heimberg, unpublished experiments), and the release of TG by the liver (1) appears to be proportional to the load of NEFA presented to it. It is quite probable that, had sufficient NEFA been added to the perfusion medium in these experiments, accumulation of TG in livers from diabetic animals would have been demonstrated.

It seems reasonable that livers from diabetic rats, in contrast to livers from normal rats or from diabetic animals treated with insulin, were utilizing hepatic triglyceride as a major source of energy. The authors reported previously that the output of ketone bodies by perfused livers from normal or acute alloxan diabetic rats was independent of the load of palmitic acid (0–80 mg) added to the medium (1). The output of ketone bodies by livers from acute diabetic rats was approximately five times that of the normal at all loads of NEFA presented to the liver. In this work, by using 120 mg of palmitic acid, the output of ketone bodies by livers from normal and controlled diabetic rats, or from the chronic diabetic rats, was in complete agreement with the earlier data. The output of ketone bodies has in the past been correlated by many workers with increased availability of NEFA to the liver (20, 21). Much of this work has been derived from experiments on intact animals, in which many direct correlations were observed between the concentration of NEFA and ketone bodies in the plasma.

The production of β-hydroxybutyrate and acetoacetate by livers from fasted rats perfused in vitro with a synthetic medium lacking fatty acid was observed to be the equivalent of 0.15 mg acetone/g liver/hr (22); when 30 mg of oleic acid/hr were infused, the rate of ketone body production was increased to about 0.40 mg acetone/g liver/hr. This rate of ketone body output was not different from that reported earlier when 90 mg of oleic acid/hr were infused (23). It has been reported also that the rate of ketone body production by rat liver homogenates was proportional to the concentration of palmitic acid in the medium (24). These discrepancies may be resolved somewhat since, in recent experiments, it was necessary to maintain the concentration of NEFA in the medium at more elevated levels than had been employed previously in order to stimulate the output of ketone bodies by the perfused liver from normal fed rats (D. R. Van Harken, C. Dixon, and M. Heimberg, unpublished experiments). It has therefore been necessary to revise upward some of the concepts concerning the capacity of the liver to metabolize NEFA.

Since the concentration of triglyceride in livers from diabetic rats is elevated, since the triglyceride appeared to be depleted during perfusion, and since the output of ketone bodies by the livers from diabetic rats exceeded that which could be supported theoretically by small loads (20 mg) of palmitic acid (1), it may be argued that endogenous hepatic triglyceride is a major substrate for ketone body formation (2,25) and that hepatic triglyceride accumulation may accompany or precede increased rates of ketone body production.

Regardless of the regulatory role that the concentration of NEFA may have on the output of ketone bodies by the perfused liver, the synthesis of ketone bodies also appears to be dependent on the nutritional state or hormonal balance of the animal from which the liver was obtained. The output of ketone bodies by the liver, for example, is stimulated by epinephrine (4,26) or glucagon (27,28) added to the medium in vitro. Ketonemia in the intact animal may then not only vary with the supply of NEFA to the liver from adipose tissue but may also vary with metabolic alterations of the ketogenic rates within the liver.

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The Renal Phospholipid Composition of Choline-Deficient Rats

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ABSTRACT

The base composition of the phospholipids involved in the N-methylation pathway for the biosynthesis of phosphatidyl choline was determined in normal and severely hemorrhagic rat kidneys. There was a decrease in the proportion of phosphatidyl choline and phosphatidyl ethanolamine in the renal total lipids. The significant decrease of phosphatidyl ethanolamine in the kidney phospholipids appears to implicate this phospholipid to a greater extent than phosphatidyl choline in the etiology of the hemorrhagic syndrome.

INTRODUCTION

SINCE THE DISCOVERY by Griffith and Wade (1) of renal hemorrhagic degeneration as one of the direct effects of a deficiency of choline in the diet of weanling rats, the relationship between the appearance of fat droplets in the proximal epithelial cells of the cortex and the biochemical aberrations involved in the degenerative pathological process remains unelucidated (2).

Baxter and Goodman (3) were unable to demonstrate an increase in renal lipids prior to the manifestation of degeneration. Patterson and McHenry (4, 5) however showed that, although the amount of total renal lipids was approximately the same for the choline-deficient and choline-supplemented animals during the pre-lesion period, both the concentration and actual amount of the kidney phospholipids had decreased in the deficient group. These phospholipid changes were also observed after the development of the lesion when the total lipid concentration in the hemorrhagic kidneys was lower than for the control animals.

Subsequent studies concerning the effect of choline deficiency on renal lipid composition confirmed the decreased concentration of total lipids and phospholipids in hemorrhagic kidneys (6). These later studies also showed that the fatty acid patterns of the total lipids from severely hemorrhagic kidneys exhibited a striking decrease in the percentage of linoleic and arachidonic acids when compared with corresponding data which were obtained from normal kidneys. This change in fatty acid composition was reflected primarily in the phospholipids and to a far lesser degree in the neutral lipids. The fatty acid changes in these studies appeared to be related to the severity of the kidney lesion caused by choline deprivation rather than to variations in the fatty acid composition of the diet. The same results were obtained whether a laboratory ration was used or whether the only dietary difference between the control and experimental animals involved supplementation of the control diet with choline.

Two pathways are known for the de novo biosynthesis of phosphatidyl choline: Kennedy's scheme via cytidine diphosphocholine (7) and Bremer and Greenberg's scheme of direct methylation of phosphatidyl ethanolamine, involving S-adenosylmethionine as methyl donor (8,9). This pathway also represents a mechanism for the de novo biosynthesis of choline (10, 11). In the rat the methylating pathway is localized mainly in the liver, where it is quantitatively important (11, 12). In vitro experiments have shown however that measurable activity of the transmethylating enzyme system is also present in rat kidney (11).

In severely hemorrhagic kidneys the rigid dietary restriction of methyl donors in the experimental animals may conceivably alter the proportion of one or more phospholipids involved in the methylating pathway.

The present studies were undertaken to determine whether the changes observed in the phospholipid fatty acid composition in severely hemorrhagic kidneys might be associated with changes in the composition of phospholipids involved in the methylating pathway. The kidney lipids of the choline-deficient animals were also examined for the presence of phosphatides containing monomethylethanolamine (MMEA) and dimethylethanolamine (DMEA), which would represent partially methylated precursors of lecithin.

EXPERIMENTAL

Male weanling rats of the Sprague Dawley strain weighing 40 to 60 g were used. During the third week of lactation, dams and their litters were fed a vitamin B_{12}-deficient diet, which was normal with respect to other nutrients and included 0.28% choline chloride. The reason for the inclusion of this diet as

well as its composition have been reported previously (6). All the animals received this preweanling diet.

During the experimental period one group of rats was fed a semipurified, choline-deficient diet known to produce severely hemorrhagic kidneys. The composition of the diet, including its fatty acid composition, was the same as that reported previously (6).

The animals in a second group were fed the same batch of laboratory ration (Purina Rat Chow, Ralston Purina Company, St. Louis, Mo.) as that used previously since this had been shown to produce the same fatty acid patterns in normal kidneys as the semipurified diet supplemented with 0.28% choline chloride (6). Both groups were given food and water ad libitum throughout the experimental period.

Treatment of the Kidneys

This was essentially the same as previously described for the preparation of the total lipid extracts (6) except that the kidneys from three animals were pooled for each sample in order to provide sufficient lipid extract for subsequent analysis to be carried out on a single sample. Correspondingly larger volumes of extracting solvent were used. The dried lipid extracts were stored in chloroform: methanol (2:1) at −15C. Suitable aliquots were removed for dry weight determinations, followed by phosphorous determinations using the Bartlett procedure (13).

Ion Exchange Chromatography

The amounts of lipid used for ion exchange chromatography ranged from 80-106 mg for the normal kidneys and from 50-90 mg for the severely hemorrhagic kidneys. The lipid extracts were hydrolyzed for 6 hr as described by Horowitz and Beadle (14). The cooled hydrolysates were extracted five times with chloroform to remove any lipid contaminants. The neutralized samples were chromatographed on Dowex-50 cation exchange columns (Microchemical Specialties Company) (15). The eluates were examined for the presence of MMEA, serine, and ethanolamine by the method of Hayashi et al. (16). Before analysis the samples were neutralized by adding 0.25 ml 6N NaOH and 0.75 ml 0.5 M Na_2 H PO_4 to 1 ml of column eluate. Aliquots of appropriate fractions containing serine and ethanolamine were then combined, neutralized, and used for quantitative determination of these compounds (16). Choline was detected by the "hanging drop" technique, using chromotropic acid (17). In addition, the eluates were tested for the presence of MMEA, DMEA, and choline by assaying aliquots with *Neurospora crassa* strain 34486 (14). Aliquots of appropriate fractions were then combined for quantitative assay of these compounds by the same procedure. (MMEA was obtained from Eli Lilly and Company; DMEA from Eastman-Kodak Company.)

TABLE I

Phosphorus, Serine, Ethanolamine, and Choline Content of Total Lipids from Normal and Severely Hemorrhagic Rat Kidneys[a]

	μMoles per 100 mg total lipid	
	Normal	Severely hemorrhagic
Phosphorus	116.2±1.7	71.0±2.6
Serine	3.2±0.4	2.5±0.8
Ethanolamine	25.4±2.1	11.9±1.4
Choline	46.9±4.0	34.3±1.5
Serine+Ethanolamine+Choline	75.5±6.1	48.7±2.8

[a]Mean of five samples, each representing tissues pooled from three animals, ± standard error of the mean.

RESULTS

There was an increase in kidney weight from 0.99 (± 0.01)% body weight for the ration-fed rats to 1.89 (± 0.08)% body weight for the severely hemorrhagic kidneys of the choline-deficient animals. The value obtained for the kidneys of the ration-fed animals was essentially the same as that found previously for both the kidneys from animals which were fed the semipurified synthetic diet, supplemented with choline, and for ration-fed animals. The increased kidney weight of the severely hemorrhagic kidneys was the same as the value previously found in choline-deficient animals (6).

Data obtained for the renal lipids (Table I) showed the typical decrease (35.5%) in the proportion of lipid phosphorus, indicating a decrease in the percentage of phospholipids in the severely hemorrhagic kidneys compared with normal kidneys. This confirms the observations in previous studies (5, 6).

The analyses for the individual phosphatide bases showed that the decreased phospholipid concentration was reflected in the decreased concentrations of ethanolamine and choline in the total lipids of severely hemorrhagic kidneys. The decrease was more marked for ethanolamine (53.1%) than for choline (26.9%). There was no significant change in serine concentration.

The proportions of these phosphatide bases in the renal phospholipids did not show large differences between the two tissues being compared (Table II). There was a statistically significant decrease of 5% in the ethanolamine

content of kidneys with lesions ($0.02 < P < 0.05$). There was no significant difference however in the proportions of choline or serine, or in the combined molar concentrations of choline, ethanolamine, and serine between the two experimental groups.

Normal kidneys showed a ratio of choline to ethanolamine concentration in the phospholipids of 2 to 1 compared with a 3 to 1 ratio observed in the kidneys with lesions (Table III). The base ratio for ethanolamine to serine was 50% greater for the normal kidneys than for the severely hemorrhagic kidneys.

No evidence was found for the presence of MMEA, DMEA, or their corresponding phosphate or phosphatide derivatives in either the normal or severely hemorrhagic kidneys under the conditions of the experiment.

As the values for serine were lower than those reported by Marinetti et al. (18), the effect of the conditions of hydrolysis on serine stability was investigated. Serine solutions were prepared which contained 2.5, 5, and 10 times the amount of serine recovered from the Dowex ion exchange columns when renal lipid hydrolysates were examined.

The samples were autoclaved and analyzed according to the same procedure used for experimental samples. No difference was obtained from control samples which were not autoclaved.

DISCUSSION

The implication of phospholipids in the etiology of renal lesions in choline-deficient weanling rats seems apparent, from the earlier findings of Patterson and McHenry (4, 5) and from confirmatory findings both previously (6) and in the present study, that there is a decreased concentration of phospholipids in rat kidneys which exhibit the hemorrhagic syndrome. This is reflected in a lower concentration of phospholipids in the renal total lipids.

The decreased concentration of choline and ethanolamine, but not of serine, in the hydrolysates from the total lipids of severely hemorrhagic kidneys indicates a lower concentration of phosphatidyl choline and phosphatidyl ethanolamine in the diseased kidneys. This, in turn, suggests a decreased rate of N-transmethylation since the CDP choline: diglyceride cholinephosphotransferase system is apparently not affected by choline deficiency (19). These results however were obtained with hepatic tissue, and this system, if present in the kidney, may be influenced by a dietary lack of choline.

TABLE II

Serine, Ethanolamine, and Choline Content of Phospholipids from Normal and Severely Hemorrhagic Rat Kidneys[a]

	μ Moles/100 μ moles lipid P	
	Normal	Severely hemorrhagic
Serine	2.9±0.3	4.2±0.5
Ethanolamine	22.5±1.8	16.7±1.8
Choline	44.2±0.5	48.6±1.7
Serine+Ethanolamine+Choline	69.6±1.3	69.5±2.9

[a]Mean of five samples, each representing tissues pooled from three animals, ± standard error of the mean.

When the proportions of choline, ethanolamine, and serine were related to the renal lipid phosphorus content, the value of 69.6% for their combined molar concentration in normal kidneys compares favorably with the value of 67.3% reported by Marinetti et al. (18). In addition, the value of 25.4% for the combined molar concentration of ethanolamine and serine per total lipid phosphorus for normal rat kidneys compares with the value of 20% reported by Collins et al. (20). Results for choline confirm those of Patterson and McHenry (4), who reported a 29% decrease in the concentration of choline in the hemorrhagic kidneys but found no difference in the average amount of choline per 100 mg phospholipid in the kidneys of choline-deficient or choline-treated rats.

Jacobi and Baumann (21) observed that the actual choline content in hemorrhagic kidneys was higher than in the controls and suggested that the symptoms of choline deficiency are caused by the lack of an unidentified essential methyl-containing compound other than choline itself. In the present study the significant 5% decrease in phosphatidyl ethanolamine concentration in the phospholipids of the severely hemorrhagic kidneys resulted in a decrease in the ratio of phosphatidyl ethanolamine to phosphatidyl serine and an increase in the phosphatidyl choline to phosphatidyl ethanolamine ratio. The hemorrhagic symptoms of choline deficiency may be related to this

TABLE III

Ratios of Phosphatide Bases in Phospholipids from Normal and Severely Hemorrhagic Rat Kidneys[a]

	Normal	Severely hemorrhagic
Ratio, Choline[b] to Serine[b]	15.9±1.7	15.1±3.1
Ratio, Ethanolamine[b] to Serine[b]	8.3±1.1	4.4±0.8
Ratio, Choline[b] to Ethanolamine[b]	2.0±0.1	3.1±0.3

[a]Mean of five samples, each representing tissues pooled from three animals, ± standard error of the mean.

[b]Concentrations were calculated as μmoles/100μmoles lipid P.

decreased concentration of phosphatidyl ethanolamine rather than to that of a methyl-containing compound, as suggested by Jacobi and Baumann.

From these data it is not possible to determine whether the decreased concentration of phosphatidyl ethanolamine in the renal lipids is a result of an increased rate of utilization, a decreased rate of synthesis, or a decreased influx from an extrarenal source. It has been suggested that the kidney may not synthesize phosphatidyl choline by transmethylation but receives it intact from the liver (12). It is conceivable that a similar mechanism operates for phosphatidyl ethanolamine and that, in the hemorrhagic syndrome, there is a decreased transport of phosphatidyl ethanolamine from the liver to the kidneys. Blumenstein (22) has reported a rise in hepatic phosphatidyl ethanolamine in choline deficiency.

Evidence for the existence of different lecithin species has been reported (23, 24). Isozaki et al. (25) have related the formation of phosphatidyl choline containing a high proportion of stearic and arachidonic acids to the N-methylation of phosphatidyl ethanolamine. A second species contains more palmitate, oleate, and linoleate. Lands (26) has suggested that different biosynthetic pathways may be involved.

A preliminary investigation was therefore carried out on the fatty acid composition of the basic phosphatides involved in the N-methylating system. The pooled renal lipids obtained from three rats with severely hemorrhagic kidneys and those from three rats fed the identical choline-deficient diet but with normal-appearing kidneys were fractionated on DEAE and silicic acid-silicate-water columns (27). The purity of the fractions was checked by thin-layer chromatography. The fatty acid compositions of the separated lipids were determined as described previously (6). The lecithin in the normal-appearing kidneys contained a lower proportion of linoleic, arachidonic, eicosapentenoic, docosatetraenoic, and docosahexenoic acids and a higher proportion of palmitic and stearic acids. Phosphatidyl ethanolamine contained a lower proportion of arachidonic, eicosatetraenoic, and docosahexenoic acids and a higher proportion of palmitic acid in the normal-appearing kidneys. Further studies however are required, using larger numbers of animals to establish the fatty acid composition of the basic phosphatides during the stages of initiation and repair of the hemorrhagic syndrome.

Although this factor could be partly responsible for the decreases observed in the proportion of linoleate and arachidonate in the phospholipid fatty acids of severely hemorrhagic kidneys, it would seem unlikely to play a major role. The rapid recovery in surviving animals, without dietary change, suggests the persistence of an adaptive system throughout the recovery period (6). The decreased phosphatidyl ethanolamine concentration in severely hemorrhagic kidneys may rather result from an increased degree of utilization for the synthesis of phosphatidyl choline and choline. As the adaptive mechanism replaced or supplemented the N-methylating system, this would enable the amount of phosphatidyl ethanolamine to return to the concentration found in normal kidneys.

In this connection, data were obtained from a limited number of "frosted" kidneys. This term was used by Griffith to describe a stage of regeneration and repair of hemorrhagic kidneys (28). The kidneys were analyzed by the same procedures and at the same time as the previous study (6). The same proportion of phospholipids (79.0%) in the total lipids and the same fatty acid composition was obtained for both the neutral lipid and phospholipid fractions of the total lipid extracts as for normal kidneys. This suggests that the phospholipid composition of recovered kidneys is the same as that of normal kidneys. This would support the hypothesis that phosphatidyl choline and, to a greater extent, phosphatidyl ethanolamine concentrations, which are decreased in the lipids of severely hemorrhagic kidneys, are the same in recovered as in normal kidneys.

Since the decrease in the proportions of linoleic and arachidonic acids in hemorrhagic kidney phospholipids appears to be related to a deficiency of choline rather than other dietary factors (6), it is conceivable that choline-deficiency in male weanling rats might result in a decrease in the percentage of phosphatidyl ethanolamine in the kidney phospholipids. This effect may be implicated in the etiology of the hemorrhagic syndrome to a greater extent than in the decreased concentration of phosphatidyl choline observed in the renal total lipids.

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Identification and Composition of Turnip Root Lipids

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ABSTRACT

Two varieties of turnip, Laurentian and Wye, were examined for their lipid and fatty acid composition. Lipids extracted with 80% ethanol contained variable quantities of phosphatidic acid, which was considered to be an artifact. Crude lipids were fractionated by TLC, and fatty acids and sterols were analyzed by GLC. Among the common phospholipids, cardiolipid and phosphatidyl glycerol were abundant components. Linolenic acid comprised 60% of the total fatty acids. β-Sitosterol was the principal sterol, and about half of the carotenoids was lycopene. No great differences between the two varieties studied were observed however.

INTRODUCTION

TURNIP IS A MEMBER of the mustard family, *Cruciferae*. Lipids of this genus are best known in cabbage (1,2) and more particularly in the oil-bearing seeds, mustard (3) and rapeseed (4,5).

Turnip roots are consumed as a vegetable and are also processed as instant food. Instant turnip has a long shelf-life, longer than instant potato, for example. It was therefore considered of interest to determine the lipid composition of turnip.

The present paper sets forth a detailed study of the lipid and fatty acid composition of two turnip varieties, Laurentian and Wye. Chromatographic techniques and identification procedures used in this study are described.

EXPERIMENTAL PROCEDURE

Materials and Extraction

Turnips, the Laurentian and Wye varieties used in this study, were grown at the Experimental Farm, Nappan, Nova Scotia, in 1964. Also Laurentian turnip was grown at the Central Experimental Farm, Ottawa, in 1965. This variety is a deep yellow turnip, indicating a high content of carotenoids, in contrast to the Wye variety, which is colorless.

Samples (100 g) were minced and homogenized for 2 min in 3 vol of chloroform: methanol (2:1). After standing for 2 hr with intermittent shaking, the homogenate was filtered and the residue was re-extracted twice with the same solvent mixture. All extracts were pooled and concentrated to dryness in a rotary evaporator under nitrogen, below 30C.

Samples of turnip flakes were also extracted by the same procedure. In addition, samples of fresh turnip were extracted with 80% hot ethanol (6) and hot isopropyl alcohol (7).

Crude lipids were taken up in chloroform: methanol (19:1) and purified through a Sephadex, G 25, column (8). Lipids were eluted with 35 ml of water-saturated chloroform:methanol (19:1). The subsequent fractions, which had no lipid, were discarded. The lipid fraction was concentrated to dryness and was taken up in chloroform:methanol (19:1) at a concentration of 25 mg/ml.

Standard mixtures containing fatty acids, triglycerides, sterol and sterol esters, or phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and cardiolipid (General Biochemicals, Chagrin Falls, Ohio) were prepared for TLC standardization. Commercial soya lecithin and egg lecithin were also used as reference material.

Column Chromatography

Turnip lipid extracts were then subjected to column chromatography, using a 0.9 cm x 13 cm, silicic acid (Bio-Sil HA–325 mesh, Bio-Rad, Richmond, Calif.) column. Samples (50 mg) were transferred onto the column in pure hexane, and the elution was started with hexane. It was continued with 2%, 5%, and 50% ether in hexane, then with 2%, 5%, 10%, and 50% methanol in chloroform. After removal of the solvent in vacuum, the lipid was dissolved in chloroform and aliquots were tested by TLC.

Elution of carotenoids was carried out with pure hexane, then with 5% and 50% ether in hexane. The four bands which separated were collected separately and tested for their homogeneity by TLC.

Thin-Layer Chromatography

Thin-layer chromatography was performed on standard 20 cm x 20 cm chromatoplates, coated with a 250 μ layer of silica gel G. The

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solvent system hexane:isopropyl ether:diethyl ether:acetone:acetic acid (85:12:1:4:1, v/v) was found to be the most suitable for the separation of neutral lipids, including carotenoids and tocopherols. The hexane:ether:acetic acid solvent system was used for the purification of fatty acid methyl esters. The solvent system (9) chloroform:acetone:methanol:acetic acid:water (5:2:1:1:0.5) was used for the separation of polar lipids. A two-dimensional system consisting of solvents chloroform:methanol:water (65:15:2), first in the x-direction, and chloroform:acetone:methanol:acetic acid:water (6.5:2:1:1:0.3) then in the y-direction was also found to be useful. Spots were located by exposure to iodine vapor or by spraying with a dichromate solution in 50% sulfuric acid.

The lipids separated by TLC were identified by comparing R_f values with those reported earlier (10) and with those of pure compounds, or by using specific sprays, such as ninhydrin, Dragendorff' reagent, or molybdic acid (11) for phospholipids and perchloric acid for glycolipids (10).

Individual lipids were isolated on a preparative scale by TLC. A guide strip containing the standard mixture was used as described (12). After development of the chromatoplate, the spots were visualized by spraying with dichlorofluorescein or by covering the plate with Saran Wrap and exposing the guide strip to iodine vapors. The desired zones were then scraped off the plate, and the lipid was eluted from the silica gel with a mixture of chloroform:methanol (1:1) and used for the GLC analysis of their fatty acids.

Certain fatty acids methyl esters were separated on 12.5% silver nitrate–silica gel plates prior to their separation by gas-liquid chromatography on a SE 30 silicone column.

Gas-Liquid Chromatography

The methyl esters of fatty acids were prepared according to the method of Morrison and Smith (13). Samples were heated in screw-cap test tubes, containing 1 ml of boron trifluoride (Applied Science Laboratories), 1 ml benzene, and 1 ml methanol at 90C for 45 min. Methyl esters were purified by TLC prior to GLC.

The instrument used for GLC was a Research Specialties Series 600 gas chromatograph, equipped with a flame detector and a 6 ft x 3/16 in.-column of 5% w/w DEGS on 60/80 mesh Gas-Chrom P, operating at 183C (14). Fatty acids were identified by comparison of their retention times with those of pure compounds (The Hormel Institute). Each methyl ester was calibrated by using 17:0 as marker and plotting the relative peak heights *vs.* the amounts injected (15).

Sterols were converted to their TMS derivatives and separated on a 3% JXR on Gas-Chrom Q, 100/120 mesh, column with the same instrument, at a column temperature of 210C. Components were identified by comparing retention times with those of known compounds (14).

Other Identification Techniques

Fractions obtained by column chromatography were deacylated with 0.2N methanolic potassium hydroxide at 37C for 20 min. The hydrophyllic portions were separated by chromatography on paper (10).

Absorption spectra of carotenoids were obtained with a Bausch & Lomb (spectronic) 502 spectrophotometer. Quantitative measurements were made with a Bausch & Lomb 340 spectrophotometer, at the maximum wavelengths, in water-saturated n-butanol.

Some carotenoid fractions were partitioned between hexane and 90% methanol (16). Others were examined for epoxides by the hydrochloric acid-ether color test (17).

Infrared spectra of phospholipid were obtained with a Perkin-Elmer Model 21 spectrometer, equipped with sodium chloride optics.

RESULTS

The total lipid content of turnip was found to be 1.9%, as calculated on dry-weight basis. Moisture content was 82% in Laurentian and 88% in Wye turnip. As determined by column chromatography, Laurentian turnip contained 61.5% polar lipid and 38.5% neutral lipid. In potato lipid this ratio was 83.5 to 16.5 (18).

Polar Lipids

In preliminary experiments it was noted that differences occurred in the composition of turnip lipids when various solvent systems were used. Three solvent systems, hot 80% ethanol followed by chloroform:methanol (2:1), hot isopropyl alcohol, and a mixture of chloroform:methanol (2:1), were tested. Results are shown in Figure 1. The ethanol extracts showed inconsistent amounts of phosphatidic acid (spot 8), which appears as an artifact. This compound was present in much smaller amounts in the isopropyl alcohol extract and negligible in the chloroform:methanol extract. In contrast, phosphatidyl glycerol and cardiolipid seem to disappear in the first two systems. Phosphatidyl inositol, phosphatidyl cho-

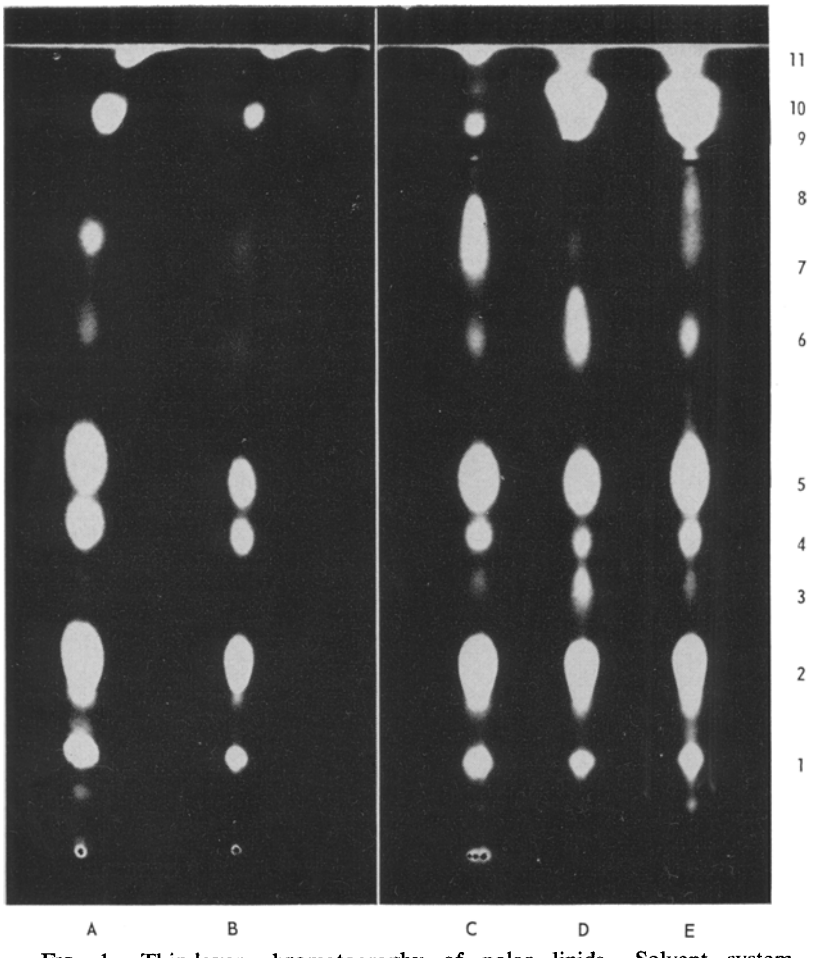

FIG. 1. Thin-layer chromatography of polar lipids. Solvent system—chloroform:acetone:methanol:acetic acid:water (5:2:1:1:0.5). Spots were detected with iodine vapors. A and B, potato lipids used as reference; C, turnip lipids extracted with 80% ethanol; D, lipids extracted with C/M (2:1); and E, lipids extracted with isopropyl alcohol. Spots were identified as: 1, phosphatidyl inositol; 2, phosphatidyl choline; 3, phosphatidyl glycerol; 4, digalactosyl diglyceride; 5, phosphatidyl ethanolamine; 6, cardiolipid; 7, sterol glucoside; 8, phosphatidic acid; 9, monogalactosyl diglyceride; and 10, neutral lipid.

line, phosphatidyl ethanolamine, and digalactosyl diglyceride show the same proportions in all extracts however. Very little phosphatidic acid was encountered in the extraction of potato lipids with 80% ethanol (18).

Fractionation of polar lipids by TLC showed similar patterns for both varieties, Laurentian and Wye. The incomplete separation of Laurentian lipids by one-dimensional TLC (Figure 1) was greatly improved by the two-dimensional techniques, as evidenced by the complete resolution of spots 2 and 3, 8 and 9 in Figure 2.

Cardiolipid

Spot 7, Figure 2, was identified as cardiolipid (diphosphatidyl glycerol) by showing it to be similar to authentic cardiolipid (General Biochemicals). After its isolation by column chromatography and purification by TLC, its infrared spectrum revealed the same absorption bands as those given for cardiolipid (19). It showed OH group absorption at 3400 cm^{-1}, strong CH_2 absorptions at 2900 and 2825 cm^{-1}, strong ester group absorption at 1735 cm^{-1}, methyl and methylene group absorptions at

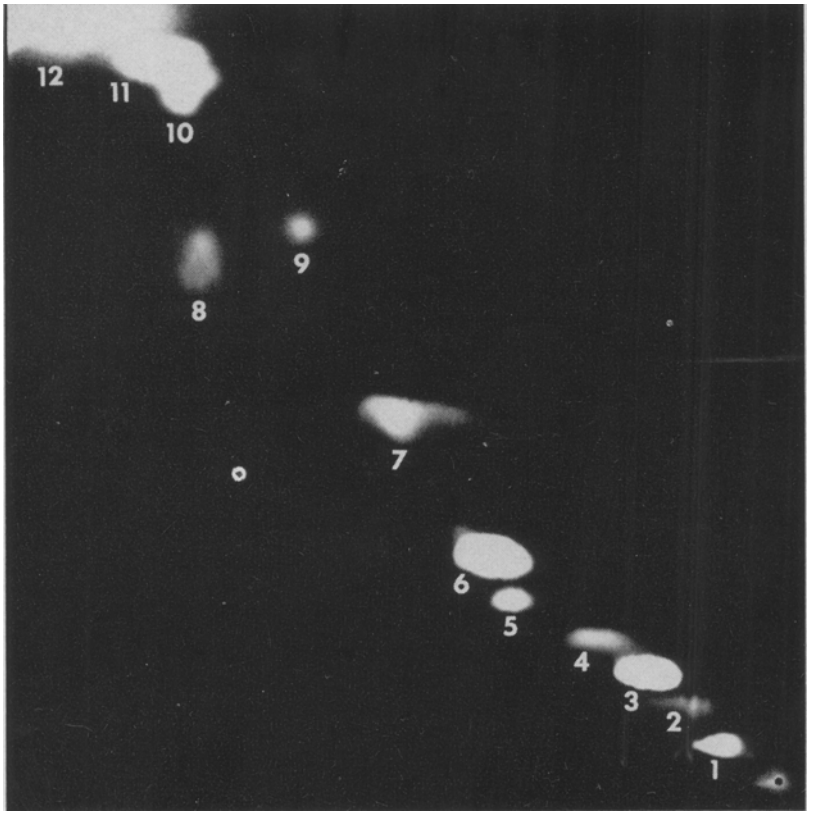

FIG. 2. Two-dimensional, thin-layer chromatography of polar lipids extracted from Laurentian turnip by chloroform:methanol (2:1). Solvent systems—chloroform:methanol:water (65:15:2) in the x-direction and chloroform:acetone:methanol:acetic acid:water (6.5:2:1:1:0.3) in the y- direction. Spots were detected with iodine vapor and identified as follows: 1, phosphatidyl inositol; 2, unidentified; 3, phosphatidyl choline; 4, phosphatidyl glycerol; 5, digalactosyl diglyceride; 6, phosphatidyl ethanolamine; 7, cardiolipid; 8, sterol glucoside; 9, phosphatidic acid; 10, monogalactosyl diglyceride; 11, free sterol and esterified sterol glucoside; and 12, triglycerides.

1460 and 1380 cm^{-1}, and P:O group absorption at 1250 cm^{-1}.

After its deacylation with 0.1N potassium hydroxide, diglycero-phosphoryl glycerol (GPG PG) was detected on paper chromatograms, as demonstrated by Benson and Strickland (20).

Cardiolipid appears to be one of the most abundant phospholipids, with phosphatidyl choline and phosphatidyl ethanolamine, as evidenced by the size of the spot in Figure 2 and by spraying with molybdic acid.

Neutral Lipids

Neutral lipids were resolved by TLC as shown in Figure 3. The components were identified with the aid of pure compounds such as sterol, fatty acid, triglyceride, fatty acid methyl ester, and sterol ester. Sterol ester, sterol, and triglyceride were the most important neutral lipids, but traces of mono- and

TABLE I
Fatty Acid Composition of Turnip Lipids
(Relative Weight Percentage)

Fatty acids	Wye	Laurentian	
16:0	15.0	13.0[a]	13.3[b]
16:1	0.7	0.8	1.1
17:1	2.6	1.4	....
18:0	1.5	1.7	0.8
18:1	7.8	7.1	9.1
18:2	13.9	18.7	19.1
18:3	58.6	57.3	56.6

[a] The 1964 crop.
[b] The 1965 crop.

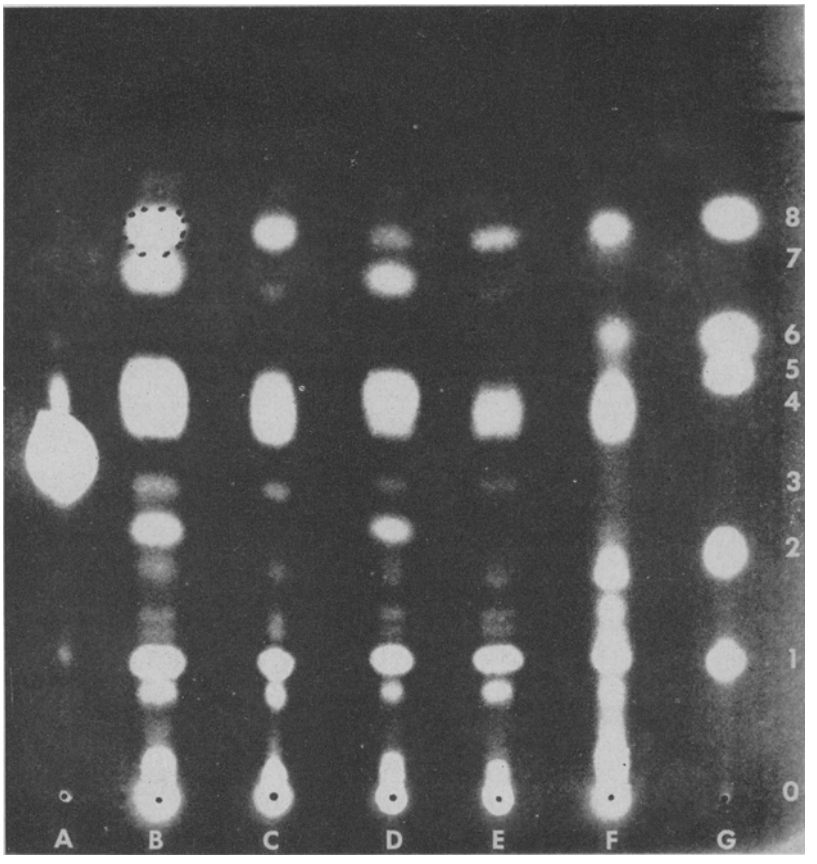

FIG. 3. Thin-layer chromatography of neutral lipids. Solvent system—hexane:isopropyl ether: diethyl ether-acetone:acetic acid (85:12:1:4:1). Spots were located with iodine vapors. A, β-tocopherol; B, turnip flake, Laurentian; C, fresh turnip, Laurentian; D, turnip flake, Wye; E, fresh turnip, Wye; F, potato lipid; and G, standard mixture. Spots of standard mixture are: 1, sterol; 2, fatty acid; 5, triglyceride; 6, fatty acid methyl ester; and 8, cholesterol ester.

diglycerides, fatty acid, and tocopherol were also present. In the lipids extracted from turnip flakes (B and D) some oxidized fats were detected. Apart from the carotenoids found in Laurentian, no great difference was encountered between the two varieties.

Fatty Acid Composition

The fatty acid composition was determined by GLC. The main fatty acids were palmitic, oleic, linoleic, and linolenic acids. The percentage composition, as calculated by the relative peak height ratio calibration method, is

TABLE II

Fatty Acid Composition of Laurentian Turnip Lipids. Total, Polar, and Neutral Lipids and Their Components[a] (Relative Weight Percentage)

Fatty acids	Total	Polar	Neutral	PC	PE	PI	DG	MG	Cardiolipid
16:0	13.3	14.2	10.9	10.2	12.6	33.6	21.2	12.1	11.3
16:1	1.1	1.3	1.2	1.0	0.7	0.7	1.8	1.1	1.3
18:0	0.8	0.9	1.2	0.8	0.9	0.8	1.2	0.9	0.8
18:1	9.1	9.3	8.8	10.7	11.3	4.0	7.7	9.6	9.0
18:2	19.1	19.6	17.0	19.6	24.9	14.6	13.5	20.4	20.8
18:3	56.6	54.7	60.9	57.7	49.7	47.1	54.7	55.8	56.9

[a] PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; DG, digalactosyl diglyceride; MG, monogalactosyl diglyceride.

TABLE III
Absorption Maxima (mμ) for Each Carotenoid Eluted from a Silicic Acid Column

Fractions	Measured spectra max mμ	Reference spectra max mμ (20)	Solvent	Carotenoid
A	441 468 499 448 473 503	441 469 500	Hexane WSB[a]	Lycopene
B	434 458 489 ..... 463 493	434 459 490	Hexane WSB	γ-Carotene
C	424 448 480 430 456 487		Hexane WSB	Unidentified
D	424 453 486 428 458 490	425 451 483	Hexane WSB	Cryptoxanthin

[a]WSB = water-saturated n-butanol.

shown in Table I. It is noted that linolenic acid is by far the most important fatty acid. A minor fatty acid, which chromatographed with the same retention time as 17:1, was also present, it was separated by silver nitrate TLC and run on a SE 30 silicone column. Both Laurentian and Wye turnips had about the same fatty acid composition. No erucic acid was detected however.

Table II gives the percentage composition of fatty acids in total lipids, in neutral and polar lipids, and also in the individual components. Linolenic acid was the major fatty acid in all of them. Palmitic acid was higher in phosphatidyl inositol and digalactosyl diglyceride than in the other components. On the other hand, linoleic acid was less in these two components.

Sterol and Sterol Glucosides

The presence of sterol, sterol esters, and sterol glucosides was shown by TLC (Figures 2 and 3). The esterified sterol glucoside found in potatoes (10,14) was also present in turnip but in smaller quantities than the free sterol glucoside. The sterol portion, after liberation by alkaline and acid hydrolysis, was analyzed by GLC as its TMS derivative and was found to consist of β-sitosterol and campesterol in the ratio of 10:1. Stigmasterol was present in trace amounts only.

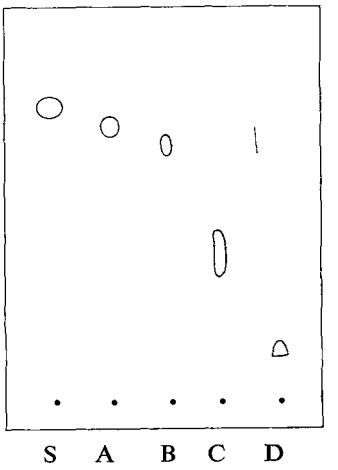

FIG. 4. Thin-layer chromatography of turnip carotenoids. Solvent system hexane:isopropyl ether:diethyl ether:acetone:acetic acid (85:12:1:4:1). S β-Carotene; A, fraction A (lycopene); B, fraction B (γ-carotene; C, fraction C (unidentified); and D, fraction D (cryptoxanthin).

Carotenoids

Carotenoids found in the Laurentian turnip were fractionated by column chromatography.

TABLE IV
Column Chromatographic Separation of Turnip Carotenoids

Fractions	Composition of eluate	Spectral absorption max mμ	Percentage composition[a]	Carotenoids
A	Hexane	472	41.9	Lycopene
B	Hexane	462	28.3	γ-Carotene
C	5% ether in hexane	456	12.3	Unidentified
D	50% ether in hexane	460	17.5	Cryptoxanthin

[a]$E^{1\%}_{1cm}$ values for lycopene (3470), γ-carotene (2720), cryptoxanthin (2460), and fraction C (cald as β-carotene).

Elution, carried out with pure hexane, 5% and 50% ether in hexane, resulted in the separation of four components. These were checked for purity by TLC (Figure 4). Their R_f values in the solvent system hexane:isopropyl ether: diethyl ether:acetone:acetic acid were 0.69, 0.64, 0.37, and 0.14 respectively. β-Carotene, which was not observed in these extracts, had a R_f value of 0.74.

Partition of these carotenoids between hexane and 90% methanol showed that they were all hypophasic, therefore not free xanthophyll. After treatment with mild alkali they were still hypophasic, which eliminated the possibility of esters. The hydrochloric acid-ether color test was negative, indicating that they were not epoxides.

Table III gives absorption maxima for each carotenoid eluted from the column. By comparison of their maxima with those reported by Goodwin (21), it was noted that fraction A had the same maxima as lycopene, that fraction B corresponded to α-carotene and fraction D to cryptoxanthin. Fraction C was not identified however.

Table IV gives relative weight percentages of carotenoids. Measurements were made at the maximum spectral absorption of each carotenoid in water-saturated n-butanol. $E_{1cm}{}^{1\%}$ values reported by Goodwin (21) were used in the calculation of percentages. The most important carotenoid was lycopene; it amounted to 42% of total carotenoids.

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A Quantitation Problem in the Open Tubular Gas Chromatography of Fatty Acid Esters from Cod Liver Lipids

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ABSTRACT

Methyl esters of fatty acids of marine origin contain large amounts of highly unsaturated long-chain fatty acids. It is shown that, although esters of saturated and monounsaturated fatty acids can be quantitatively analyzed on open tubular columns with a flame ionization detector, there are serious losses of the long-chain highly unsaturated fatty acids of marine oils on the column. Through comparison of chain-length composition and iodine value some correction factors are suggested for the highly unsaturated fatty acids which permit reasonably accurate analyses.

INTRODUCTION

THE PROBLEM of assuring accurate quantitation of analyses of esters of fatty acids by gas-liquid chromatography is one of continuing interest as new types of apparatus, column packings, detectors, and other variations in technology are introduced (1). Comparisons of proportions of standards usually produce adequate results in the hands of experienced operators although, even in these surveys, surprising results are sometimes observed (2, 3).

It has been reported that satisfactory quantitation can be achieved with commercial gas chromatographic equipment, employing open tubular columns, an injection splitter system, and a flame ionization detector (4, 5). In initial work with a Perkin-Elmer Model 226, using a BDS (butanediolsuccinate polyester) coated open tubular column, these reports were verified with an NIH reference mixture of saturated acids (Table I). Subsequently apparently satisfactory quantitation of methyl esters of fatty acids from rapeseed oil was achieved, as indicated by agreement of calculated and experimental iodine values, and of chain-length proportions with those obtained by hydrogenation of the sample (6). The monoethylenic fatty acids from herring oil were studied with both open tubular and packed columns, and good comparative analyses were obtained (7). In all of these experiments appropriate flame ionization response corrections were employed (8). However these materials lacked any significant proportion of long-chain highly unsaturated fatty acids. The levels of triethylenic C_{18}, C_{20}, and C_{22} acids in the rapeseed oil esters were too low to have any recognizable effect on the iodine value or chain-length calculations.

In marine lipids the longer-chain polyunsaturated fatty acids are particularly prominent components. Unexpectedly some comparisons of analyses of fatty acid esters from cod liver oil on the open tubular column with analyses on conventional packed columns in other apparatus showed serious losses of the C_{20} and C_{22} polyunsaturated components on the open tubular column. This report is concerned with a detailed study of the scope of the problem.

EXPERIMENTAL

All open tubular columns were BDS, 150 ft in length, and 0.01 in internal diameter. Operating conditions in a Perkin-Elmer Model 226 were column temperature 170C, injection port temperature 260C, No. 2 splitter installed, carrier gas helium (Union Carbide of Canada Ltd., Purified grade) at 40 psig. Peak areas were determined with a Disc Instruments Inc.,

TABLE I

Results of Two Analyses of NIH Quantitation Standard F (2). Weight Percentage from Peak Areas Corrected for Flame Ionization Detector Response (8)

Fatty acid	Known weight percentage	Gas-liquid chromatographic results	
		Analysis A	Analysis B
14:0	2.5	2.5	2.5
16:0	4.2	5.1	4.4
18:0	7.3	7.7	7.7
20:0	13.6	12.4	13.5
22:0	25.4	24.8	25.7
24:0	47.0	47.6	46.2

TABLE II
Results of Cod Liver Triglyceride Fatty Acid Analysis (as Weight Percentage) Before and After Revision

Fatty acid	*r*18:0	Complete analysis[a]			Chain-length totals	
		From peak areas[b]	Column correction factor	After revision[c]	From complete analysis	From hydrogenated sample
12:0	0.112	0.05	...	0.04	0.04	0.06
13:0	0.165	0.02	...	0.02	0.02	0.02
14:0	0.240	3.59	...	3.27	3.32	3.16
A	0.258	0.09	...	0.08	0.08	0.17
14:1ω5	0.275	0.06	...	0.05	...	...
Iso 15:0	0.290	0.23	...	0.21	0.21	0.19
Anteiso 15:0	0.310	0.06	...	0.05	0.05	0.05
15:0	0.342	0.34	...	0.31	0.31	0.38
Iso 16:0	0.415	0.10	...	0.09	0.09	0.09
B	0.449	0.06	...	0.05	0.05	0.04
16:0	0.488	9.80	...	8.94	20.82	20.10
C	0.525	0.28	...	0.26	0.26	0.20
16:1ω9	0.538	0.56	...	0.52	...	...
16:1ω7	0.555	10.86	...	9.90	...	...
16:1ω5	0.583	0.28	...	0.26	...	...
Iso 17:0	0.600	0.18	...	0.16	0.16	0.15
Anteiso 17:0 + 16:2ω7	0.633	0.10 + 0.02	...	0.08 + 0.02	0.08	0.10
16:2ω4	0.680	0.23	...	0.21	...	...
D + 17:0	0.700	0.25 + 0.46	...	0.23 + 0.40	0.23 + 0.71	0.20 + 0.66
16:3ω6	0.765	0.06	...	0.05	...	...
17:1ω8	0.770	0.34	...	0.31	...	...
16:3ω4	0.810	0.34	...	0.31	...	...
Iso 18:0	0.845	0.10	...	0.11	0.11	0.08
16:3ω3	0.850	0.05	...	0.04	...	...
16:4ω3	0.948	0.02	...	0.02	...	...
18:0	1.00	2.26	...	2.06	28.11	28.69
16:4ω1	1.02	0.34	...	0.31	...	...
18:1ω9	1.08	17.82	...	16.28	...	...
18:1ω7	1.13	5.94	...	5.41	...	...
18:1ω5	1.19	0.23	...	0.21	...	...
18:2ω6	1.33	2.49	...	1.81	...	...
19:0	1.44	0.12	...	0.11	0.24	0.26
18:3ω6 + 19:1ω10	1.53	0.02 + 0.02	1.05	0.02 + 0.02	...	...
19:1ω8	1.57	0.02	...	0.02	...	...
19:1ω7	1.60	0.02	...	0.02	...	...
19:1ω6	1.65	0.02	...	0.02	...	...
19:1ω5?	1.69	0.06	...	0.05	...	...
18:3ω3	1.73	0.79	1.05	0.77	...	...
19:?	1.88	0.06	...	0.05	...	...
18:4ω3	1.96	1.47	1.1	1.48	...	...
20:0	2.06	0.09	...	0.08	23.71	23.38
20:1ω11	2.18	2.77	...	2.52	...	...
20:1ω9	2.21	11.32	...	10.37	...	...
20:1ω7	2.28	0.28	...	0.26	...	...
20:1ω5	2.41	0.12	...	0.10	...	...
20:3ω9?	2.66	0.12	...	0.13	...	...
20:2ω6	2.70	0.28	...	0.32	...	...
21:0	2.92	NSA	...	NSA	0.31	0.37
20:3ω6	3.03	0.06	...	0.07	...	...
20:4ω6	3.29	0.27	1.2	0.29	...	...
20:3ω3	3.48	0.09	1.2	0.10	...	...
20:4ω3	3.88	0.33	1.3	0.39	...	...
20:5ω3	4.21	7.20	1.4	9.18	...	...
22:0	4.25	NSA	...	NSA	20.61	20.92
22:1ω13 + 22:1ω11	4.38	7.35		6.70		
22:1ω9	4.49	1.10	...	1.00	...	...
22:1ω7	4.67	0.12	...	0.15	...	...
21:5ω2?	6.15	0.17	1.5	0.23	...	...
21:6ω2?	6.56	0.06	1.5	0.08	...	...
22:4ω6	6.65	0.12	1.4	0.14	...	...
22:5ω6	7.32	0.14	1.6	0.18	...	...
22:5ω3	8.47	1.33	1.6	1.93	...	...
24:0	8.80	NSA	...	NSA	0.33	0.83
24:1ω9?	9.05	0.37	...	0.33	...	...
22:6ω3	9.37	6.43	1.8	10.52	...	...

[a] NSA = no significant amount.
[b] Detector correction applied.
[c] Both detector and column corrections applied.

Model 227 integrator fitted on a Honeywell ElectroniK 16 (1 mv) recorder. Attenuation changes were kept within the range 2x to 50x. All injections of samples were by Hamilton 701 syringe, with materials as solutions in petroleum ether and a back-up slug of 0.001 ml petroleum ether to flush the syringe. Auxiliary analyses were carried out on a Wilkens/Varian Hy-FI with similar recording equipment. The column in this apparatus was 6 ft

in length and ⅛ in outside diameter, stainless steel, packed with 3% EGSP-Z oganosilicone polyester (9).

The liver was removed from a healthy cod (*Gadus morhua*) and extracted by the method of Bligh and Dyer (10). It should be noted that some additional water should be added in this procedure when applied to obviously fatty livers. The lipid recovery was 36%. Part of the lipid was separated into phospholipids and triglycerides, and these were transesterified with BF_3–MeOH (11). Parts of the recovered esters from the respective lipids were used for gas-liquid chromatographic studies, and the remainder was hydrogenated (12). Sufficient esters from the triglycerides were used to obtain an iodine value of 152 ± 1 from the hydrogenation, but the hydrogenation of the esters from the phospholipids was qualitative only. The minor components were identified by comparison of hydrogenated and unhydrogenated samples and the use of plotting and separation factor procedures.

RESULTS

Preliminary experiments, using marine oil esters with various cylinders of helium and two columns other than the one employed in this study, established that losses of 22:6ω3[1] were about 50% in all experimental combinations of these potential variables.

The results of a detailed analysis of the freshly prepared esters recovered from the cod liver triglycerides are given in Table II. The presentation of data to two decimal places does not imply this order of accuracy and is done solely to show the relative proportions of minor components although there may be large errors in quantitating these minor fatty acids.

It must be stressed that no significant unsaturated components were observed in the analyses carried out for this study which could not be satisfactorily accounted for on the basis of recognized biochemical patterns in fatty acids (13).

The iodine value calculated from the area percentage responses for the various components, corrected for detector response (8), was 132 as against 152 for the experimental value. The chain-length totals were also seriously in error when compared with the results from the hydrogenated sample.

In marine fish depot fats there is a significant distribution with chain length of highly unsaturated fatty acids. There are virtually none in the C_{14} acids, commonly less than 2% in the C_{16} acids, and usually less than 5% in the C_{18} acids of which about half may be 18:3ω3 and 18:4ω3. The C_{20} fatty acids are characterized by 20:5ω3 in a proportion greater than the total of other polyunsaturated fatty acids of this chain length by approximately an order of magnitude, and the same is true of 22:5ω3 and 22:6ω3 in the C_{22} fatty acids. Previous work had shown the possibility of losses of polyunsaturated fatty acids through oxidation on metal columns or other metal parts (14, 15). This was assumed to be the case in the present study, and the further assumption was made, based on the NIH standard (Table I) and monounsaturated acid studies (6, 7), that little or no loss of saturated and monounsaturated fatty acids would occur. On this basis the C_{14} and C_{16} fatty acids could be used as internal standards to calculate the approximate losses of the longer-chain fatty acids of high degree of unsaturation.

The percentages of the C_{14} and C_{16} fatty acids obtained from corrected area measurements of the total analysis (Table II) required conversion to a figure comparable with the corresponding chain lengths indicated by the analysis of the hydrogenated methyl esters. The semiarbitrary factor of 0.9 (Table III) gives a reasonable correlation for these two chain lengths (Table III, column two) if allowance is made for analytical errors and a small loss of polyunsaturated C_{16} fatty acids.

The saturates and monounsaturated fatty acids in each of the C_{18}, C_{20}, and C_{22} chain lengths were similarly multiplied by 0.9 to give the other normalized totals of column two. The differences between the chain-length totals from the hydrogenated sample and from the normalized total saturated and monounsaturated fatty acids should then be attributable to the polyunsaturated fatty acids in each chain length (Table III, column three).

The unrevised percentages of polyunsaturates in each chain length (Table II) may be normalized by the same factor (0.9) to give the percentage figures in column four of Table III. By using the figures in columns three and four, it is then possible to calculate the percentage of polyunsaturated fatty acids in each chain length which have passed unaltered through the open tubular column (Table III, column five). The estimated losses were nearly proportional to the increases in retention times for the major polyunsaturates in each chain length. Respective figures for 18:4ω3, 20:5ω3, and

[1] Shorthand notation for chain length:number of double bonds, and inclusive carbon atoms from ultimate double bond to terminal methyl group.

TABLE III

Calculation of Recovery of Polyunsaturates by Comparison of Hydrogenation Data with Saturated and Monounsaturated Fatty Acid Proportions

Chain length	Percentage from hydrogenated sample	Normalized total of saturates and monounsaturates	Polyunsaturates by difference	Normalized observed polyunsaturates	Percentage polyunsaturates recovered
C_{14}	3.2	3.3	(–0.1)	...	...
C_{16}	20.2	19.3	0.9	1.0	...
C_{18}	28.7	23.6	5.1	4.3	85
C_{20}	23.4	13.1	10.3	7.5	73
C_{22}	20.9	7.7	13.2	7.2	55

22:6ω3 were: retention times 1.96, 4.21, and 9.37; losses of C_{18}, C_{20}, and C_{22} polyunsaturated materials 15%, 27%, and 45%. The oxidation rate for polyunsaturated fatty acids is roughly proportional to the number of double bonds except for mono- and diunsaturated acids (see below). It was therefore reasonable to assume that, if losses of the different polyunsaturated fatty acids of a given chain length occur on the column, a major part of any correction should be based on time of exposure. With this in mind the large peaks for 20:5ω3 and 22:6ω3 from the Hy-FI analyses were used as a guide for developing empirical correction factors for the open-tubular analysis. Direct conversion was not

TABLE IV

Results of Cod Liver Phospholipid Fatty Acid Analysis After Revision

Fatty acid	Complete analysis weight percentage[a] after revision[b]	Chain-length totals from complete analysis	(Weight Percentage) from hydrogenated sample
14:0	1.09	1.09	0.97
A	0.04	0.04	0.03
Iso 15:0	0.12	0.12	0.11
Anteiso 15:0	0.02	0.02	0.01
15:0	0.32	0.32	0.34
B	0.02	0.02	0.01
16:0	14.51	16.60	16.47
C	0.26	0.26	0.22
16:1ω9	0.24	...	...
16:1ω7	1.73	...	...
16:1ω5	0.12	...	...
Iso 17:0	0.24	0.24	0.30
Anteiso 17:0	0.12	0.12	0.15
D	0.48	0.48	0.41
17:0	0.69	0.81	0.85
17:1ω8	0.12	...	...
Iso 18:0	0.12	0.12	0.15
18:0	5.71	21.80	21.92
18:1ω9	9.14	...	...
18:1ω7	4.58	...	...
18:1ω5	0.55	...	...
18:2ω6	1.10	...	...
19:0	0.18	0.18	0.46
18:3ω6	0.06	...	...
18:3ω3	0.30	...	...
18:4ω3	0.36	...	...
20:0	0.12	22.34	22.14
20:1ω11	0.30	...	...
20:1ω9	4.72	...	...
20:1ω7	1.02	...	...
20:1ω5	0.12	...	...
20:2ω6	0.48	...	...
20:3ω6	0.03	...	...
20:4ω6	3.65	...	...
20:3ω3	0.30	...	...
20:4ω3	0.51	...	...
20:5ω3	11.09	...	...
21:0	NSA	0.10	0.29
22:0	NSA	35.80	34.23
22:1ω13	1.73	...	...
22:1ω11			
22:1ω9	0.42	...	...
22:1ω7	0.06	...	...
21:5ω2?	0.06	...	...
21:6ω2?	0.04	...	...
22:4ω6	0.03	...	...
22:5ω6	0.26	...	...
22:5ω3	1.86	...	...
24:0	NSA	0.42	0.67
24:1ω9?	0.42	...	...
22:6ω3	31.44	...	...

[a] NSA = no significant amount.

[b] Both detector and column corrections applied.

practical for all components since only the large peaks in two analyses were accurate enough for reliable comparison. The factors listed in Table II were applied to appropriate percentages based on areas corrected for detector response with the results shown as "after revision." The revised results gave a calculated iodine value of 155, in near agreement with the experimental 152. The chain-length proportions were also now substantially in agreement with the hydrogenated values (Table II).

Normally the fatty acids derived from marine phospholipids have somewhat different compositions from any associated triglycerides (16). The iodine values of the former are characteristically in the range 220–250 (17). Octadecenoic acids are the significant monoethylenic fatty acids, and the level of 20:4ω6 is about ten times that found in marine depot fats. The open tubular gas-lipid chromatography of the methyl esters of the fatty acids from the cod liver phospholipids gave an analysis again showing gross errors when compared with the chain-length data from the hydrogenated sample. The same column factors which were applied to the cod liver triglycerides were used to obtain the revised analysis given in Table IV. With the exception of the C_{22} chain length, the larger even chain lengths are in excellent agreement. The difference in the C_{22} chain length is not excessive for analyses of this type even if conditions not requiring extensive corrections were employed.

The presence of a high proportion of 20:4ω6 in this sample permitted a mutual comparison of the suitability of the column factors for 20:4ω6, 20:5ω3, and 22:6ω3 by comparison of the Hy-FI and Perkin-Elmer results. Agreement was better than $\pm$ 3% in all cases.

DISCUSSION

Gas-Liquid Chromatography

The losses of the more highly unsaturated fatty acids are probably caused by oxidation of the very small samples by the trace amount of oxygen (normally about 1 ppm) in the helium carrier gas (14). The metal of the open tubular column may also be involved (15). Radioactive fatty materials have been shown to be retained temporarily or permanently on polyester packed columns, presumably because of interaction with polyester and/or support (18–20). Other workers have reported opposite results (21, 22). If these losses were caused by interesterification with the polymer, then esters of saturated and monounsaturated fatty acids would be affected equally with polyunsaturated fatty acids. In fact, it has been reported by Seher that esters of saturated fatty acids were not affected whereas up to 17% of the ester of 18:1ω9 was irreversibly adsorbed by a particular column, and that another column showed losses of 22, 24, and 39% respectively for 18:1ω9, 18:2ω6, and 18:3ω3 (20).

The latter observation is in agreement with the view that the susceptibility of unsaturated fatty acids to "oxidation" is not directly related to the number of double bonds unless these number three or more. Experimental studies in this regard depend on the type of measurement employed (23) and on the mode of exposure and/or other factors (24), but in broad terms the results of a literature survey indicate that, if 18:1ω9 is taken as the basis for susceptibility to autoxidation, then 18:2ω6 is only 2 to 3 times as susceptible but 18:3ω3 is some 20 to 30 times as susceptible (23–29).

Autoxidation studies on more highly unsaturated fatty acids such as 20:4ω6, 20:5ω3, and 22:6ω3 suggest that these are more susceptible than 18:3ω3 in proportion to the number of double bonds (27). The quantitative gas-liquid chromatography of esters of these longer-chain fatty acids has recently been investigated in detail by Gerson et al. with a variety of column packings (30). The results showed wide variations in responses for the same fatty acids with different columns, but for a column of 20% ethylene glycol adipate polyester on siliconized 30–80 mesh Celite operated with an argon ionization detector at 205–206C the correction factors relative to 18:0 were respectively 18:3ω3, 1.03; 20:4ω6, 1.58; 20:5ω3, 1.72 and 22:6ω3, 3.03. These, with the exception of the factor for 22:6ω3, are nearly proportional to the empirical factors proposed for the open-tubular analysis of cod lipid fatty acids (Table II). The inclusion in the latter of retention time as a major consideration, as well as degree of unsaturation, seems justified by these experimental results.

With thermal conductivity detectors, appropriate corrections are necessary for detector response for methyl esters of fatty acids as for other compounds (2, 20). Despite the widespread use of argon ionization detectors there are few reports of erratic quantitation results for fatty acids of differing degrees of unsaturation (31–34). Possibly oxygen present in the argon at modest concentrations can cause oxidative scission (35) of oxidation-susceptible polyunsaturated fatty acids; the fragments then

give high results (33, 34). Alternatively oxygen could induce polymerization, with low results (32).

The flame ionization detector has given accurate results with mixtures rich in 18:3ω3 (8, 36) except where oxidation was involved (14). There seems to be no accurate report of anomalous flame ionization detector responses associated with ethylenic double bonds excepting one of a rather large relative response difference between decane and 1-decene (37). Possibly this is a structurally unique case related to a mode of scission in the flame, yielding formaldehyde as a primary product from 1-decene.

In one study of the response of fatty acids in flame ionization detectors to different fatty acids, some lower responses of 18:2ω6 and more extreme losses of 18:3ω3 relative to 16:0 were shown to decrease further with the diminishing sample size (38). The correlation of these losses with those of 18:0 and 18:1ω9 discussed by the authors is very rough, and it seems likely that on-column oxidation of the more unsaturated acids was at least partially involved.

Particular attention is drawn to the dangers inherent in assuming that quantitation in gas-liquid chromatography is justified for all classes of compounds if only one class, such as esters of saturated fatty acids, is verified as to accuracy in the particular analytical system. Losses of methyl esters of fatty acids in an injection splitter system or in an open-tubular column can be differentiated from detector malfunction or abuse with the aid of hydrocarbons (39, 40).

The column correction factors applied appear to be satisfactory for most types of marine lipids on the particular combination of equipment, operating conditions, and materials used in the present study. Other types of samples and other equipment or operating conditions would require adjustment of the column factors (30). Preferably these should be determined by the use of authentic reference fatty acid esters and combined with an internal standard technique. It would still be highly desirable to compare the analytical composition derived from the use of any column factors with both iodine value and chain length although the small amounts of materials commonly available from modern lipid separation techniques, such as thin-layer chromatography, would probably permit only the chain-length comparison.

The use of corrections for flame ionization detector response with the saturated and monounsaturated esters, also in hydrogenated samples, is desirable to improve accuracy among these components (8). However these corrections are negligible in comparison with the column factors for the polyunsaturated materials and may be regarded as included in the column factors.

Inherently the use of *ad hoc* correction factors is undesirable, and attention is therefore being directed to means of overcoming the actual loss of polyunsaturated esters. Complete elimination of oxygen from the carrier gas through physical purification (41) or combustion elimination of oxygen and maintenance of a reducing atmosphere by use of a hydrogen-helium mixture (42) are possibilities. In either case the addition of an antioxidant to the liquid phase may be helpful (42). Glass columns should also be considered (43). The effects of temperature and pressure programming remain to be investigated, as do the changes in retention time with column age (44).

It can be concluded that there is evidence to support the view that the fatty acids with three or more double bonds are much more susceptible to oxidation than those with one or two double bonds. Moreover, if the former are partially oxidized in open tubular gas chromatography, any column-effect corrections must be based on both the number of double bonds and on the relative retention times.

Cod Liver Lipids

The liver was obtained from a normal healthy cod of medium size. It has recently been shown that wide variations can be expected in the fatty acid composition of livers from individual cod (45). The flesh lipids, which are essentially phospholipids with little (~2%) triglyceride (46), have a remarkably constant composition except in very large fish (45). It is not known if this is also true of the liver phospholipids. Essentially the results of the present analysis indicate a fatty acid composition similar to the flesh phospholipids (16, 45, 47) but with some differences suggestive of the liver triglycerides, such as the inclusion of larger amounts of monoethylenic fatty acids. However it may be noted that the proportions of the various monoethylenic isomers within each chain length differ in the triglycerides and phospholipids. These results are part of a larger study of cod lipid and fatty acid metabolism, and the significance of this and other observations on the fatty acid composition will be published elsewhere.

The fatty acids designated A, B, and D are branched chain acids as previously reported (48). Other workers have shown that marine oils contain 4,8,12-trimethyltridecanoic, 2,6,10,-14-tetramethylpentadecanoic, and 3,7,11,15-tetramethylhexadecanoic acids (49). It was established through authentic materials (courtesy of R. P. Hansen, Wellington, New Zealand) that B was coincident with the ester of 2,6,10,14-tetramethylpentadecanoic acid and D with that of 3,7,11,15-tetramethylhexadecanoic acid. It was then possible to identify A as methyl 4,8,12-trimethyltridecanoate by calculation of retention times from structural considerations (50). In the analysis of esters from the cod liver triglycerides peak D did not separate from 17:0 and was determined on a nonpolar column (48). In the analyses of the esters from the phospholipids D appeared before 17:0, permitting quantitative determination on the polar column. As the column aged D appeared after 17:0. Trace amounts of *Iso* 14:0 and possibly of *Anteiso* 16:0 (the latter would nearly coincide with B) have not been included. The nature of component C has not been established. The doubtful components at *r* 1.69, 2.66, and 6.56 are not quantitatively significant. Only one 24:1 isomer was observed, and polyunsaturated C_{24} acids were not determined (51–53).

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Eicosenoic Acid and Other Fatty Acids of *Sapindaceae* Seed Oils[1]

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ABSTRACT

The seed oils of 11 species of *Sapindaceae* were examined, and their fatty acid composition was determined. *cis*-11-Eicosenoic acid was identified as the major fatty acid of *Koelreuteria paniculata*. It was present in nine of the 11 species in amounts from 8–60% of the total fatty acids and is evidently a common component of oils of this plant family. Arachidic acid was present in amounts up to 11%. Only three of the oils had acids of chain length greater than C–20. Seed oils of certain species of *Koelreuteria* and *Cardiospermum* are good potential sources of 11-eicosenoic acid.

INTRODUCTION

MANY SPECIES of the *Sapindaceae* family have seeds that are rich in glyceride oil, and some are utilized commercially in the countries of origin. Early analyses by Hilditch and others revealed the presence of considerable amounts of arachidic and small amounts of an eicosenoic acid in some of the oils (1). Later the seed oil of *Cardiospermum halicacabum* was found to have *cis*-11-eicosenoic acid as its major fatty acid. It amounted to 42% of the total acids (2).

In the present work 11 additional species have been examined to determine whether eicosenoic acid is of widespread occurrence in the *Sapindaceae*. The percentage composition of the fatty acids of each oil was determined by gas-liquid chromatography (GLC) of the methyl esters. The oils were also examined by ultraviolet (UV) and infrared (IR) spectroscopy and, in one instance, by fractional distillation to provide further data on their composition. Older methods of studying glyceride oils were inadequate to distinguish eicosenoic acid from other monoenoic acids.

EXPERIMENTAL

Seeds were obtained from research institutes and from established commercial suppliers. Large seeds were hulled, and the hulls, or shells, were discarded. Small seeds were ground as received. The finely-ground kernels or seeds were extracted in a Soxhlet with petroleum ether. The oils were converted to methyl esters by transesterification in methanol with hydrogen chloride catalyst. Ultraviolet spectra were determined in cyclohexane solution and infrared spectra in carbon disulfide.

The oil yield and properties of the oils are given in Table I. The classification into tribes is that of Engler (3).

There was considerable variation in oil content, even among species of the same genus, e.g., *Sapindus*. A few species of the family, such as *Litchi chinensis*, are known to have a low oil content. *Paullinia cupana* and *Harpullia pendula* appear to belong to this group. The oils of the family are classed as nondrying, and this fact is reflected in their relatively low refractive indices.

The UV and IR spectra of the oils showed no definite evidence of conjugated unsaturation or of unusual functional groups although *Koelreuteria paniculata* oil gave some weak bands in the infrared spectra which were not identified. The estimated fatty acid composition, as determined by GLC, is given in Table II. Each figure is the mean of three determinations.

Analysis of the methyl esters by GLC was carried out with a thermal-conductivity detector and with two different liquid phases: diethyleneglycol succinic acid polyester (DEGS) and silicone oil (SE–30). Identification of each acid was made by comparing its retention time with that of a reference sample under the same conditions on the same day. The composition of the esters was estimated by measuring the peak areas. Arachidate emerged as a single peak from the DEGS column, but eicosenoate and linolenate formed a combined peak. Arachidate and eicosenoate gave a combined peak on the SE–30 column. The areas were then calculated as follows:

$$\underset{\text{SE–30}}{\text{peak (20:0 + 20:1)}} - \underset{\text{DEGS}}{\text{peak 20:0}} = \text{20:1}$$

$$\underset{\text{DEGS}}{\text{peak (20:1 + 18:3)}} - \text{20:1} = \text{18:3}$$

[1]N. R. C. No. 9537.

TABLE I
Seed Oils of Sapindaceae

Tribe and species	Wt. of kernel (% of seed)	Oil, wt. % in seed	Oil, wt. % in kernel	Refractive index n_D^{25}	Iodine value (Wijs)
Koelreuterieae					
Koelreuteria paniculata Laxm.	—	22.2	—	1.4765	87.4
Koelreuteria bipinnata Franch.	69		49.4	1.4711	83.2
Dodoneae					
Dodonea viscosa Jacq.	—	17.9	—	1.4717	121.9
Dodonea lanceolata F. Muell.	—	15.4	—	1.4714	118.9
Harpullieae					
Xanthoceras sorbifolia Bunge	36	—	50.3	1.4706	116.3
Harpullia pendula Planch.	—	0.5	—	—	—
Paullinieae					
Paullinia cupana H.B. and K.	73	—	2.1	1.4704	81.0
Cardiospermum canescens Wall.	—	34.6	—	1.4704	84.7
Cardiospermum grandiflorum Sw.	—	37.5	—	1.4684	75.9
Sapindeae					
Sapindus mukorossi Gaertn.	21	—	31.3	1.4693	88.4
Sapindus drummondii H. and A.	65	—	55.8	1.4688	88.8
Sapindus saponaria L.	37	—	20.0	1.4679	76.9

The methyl esters prepared from the oil of *Koelreuteria paniculata* were distilled through a spinning band column. The fraction distilling at 151–155C at 0.5 mm. was almost pure methyl eicosenoate (n_D^{25C} 1.4530). It was hydrolyzed, and the acid was treated with alkaline permanganate (4). The product was *erythro*-11,12-dihydroxy-eicosanoic acid, m.p. and mixed m.p. 129–130C, proving that the original substance was *cis*-11-eicosenoic acid.

The weights of the C–16, C–18, and C–20 fractions from the distillation, and their composition (by GLC) were in agreement with the analysis of the total esters by GLC.

The composition of the fatty acids of *Sapindus mukorossi* (Table II) was in reasonably close agreement with the analysis by Earle and co-workers (5), which was done by the alkali-isomerization method.

DISCUSSION

Eicosenoic acid occurs in nearly all of the oils that were studied; the only exceptions were the two species of *Dodonea*. Large proportions of eisosenoic acid were found in two species of *Koelreuteria* (46 and 60%) and in two species of *Cardiospermum* (44 and 48%). The *C. halicacabum* oil previously analyzed (2) was similar to these two in composition. Three species of *Sapindus* had 13, 15, and 17% of eicosenoic acid. Thus there is a considerable degree of uniformity within each genus, and the figures for fatty acid composition support the botanical classification to that extent. However *Harpullia pendula* and *Paullinia cupana* differ markedly from the other taxa within the tribes because of their low oil content (Table I). The composition of the *Paullinia* oil resembles that of *Sapindus* sp. rather than that of *Cardiospermum* sp.

Arachidic acid was found in nearly all species but in small amounts except in *Cardiospermum* (10 and 11%). Linoleic is highest in *Dodonea* species, where eicosenoic is absent.

Only three of the oils had an appreciable amount of C–22 acids (3–4%). The quantity of erucic acid is thus small or negligible in the oils of the group. In this respect the *Sapindaceae* apparently differ from *Cruciferae*

TABLE II
Estimated Fatty Acid Composition

Species	Fatty acid, wt. % of total acids 16:0[a]	18:0	18:1	18:2	18:3	20:0	20:1	22
Koelreuteria paniculata	6	1	32	13	1	1	46	—
Koelreuteria bipinnata	7	—	21	8	—	1	60	3
Dodonea viscosa	11	3	23	59	—	4	—	—
Dodonea lanceolata	13	3	24	55	—	5	tr[b]	—
Xanthoceras sorbifolia	6	1	33	48	tr	—	8	4
Paullinia cupana	12	5	53	12	2	4	12	—
Cardiospermum canescens	3	1	18	7	12	11	44	4
Cardiospermum grandiflorum	4	1	27	7	3	10	48	—
Sapindus mukorossi	5	1	54	14	6	5	15	—
Sapindus drummondii	5	tr	55	16	4	3	17	—
Sapindus saponaria	5	4	65	6	—	7	13	—

[a] The figures represent chain length and number of double bonds.
[b] tr = trace.

oils, most of which contain erucic acid in excess of the content of eicosenoic acid. The *Koelreuteria* and *Cardiospermum* species constitute good sources of 11-eicosenoic acid.

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Optically Active Trihydroxy Acids of *Chamaepeuce* Seed Oils[1]

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ABSTRACT

Two trihydroxy acids have been isolated from *Chamaepeuce afra* (Jacq.) DC. seed oil and identified as (+)-*threo*-9,10,18-trihydroxyoctadecanoic (phloionolic) acid (9%) and (+)-*threo*-9,10,18-trihydroxy-*cis*-12-octadecenoic acid (14%). The unsaturated acid has not previously been found in nature. Nuclear magnetic resonance, infrared, thin-layer chromatography, optical rotation, and identification of the oxidative cleavage products show that these two trihydroxy components have the structures indicated. *Chamaepeuce hispanica* DC. seed oil and the seed oil of an unidentified *Chamaepeuce* species apparently contain these same two acids but in different proportions from *C. afra* oil.

INTRODUCTION

PUBLISHED INFORMATION concerning hydroxylated fatty acids from plants has been reviewed by Downing (1). The 9,10,18-trihydroxyoctadecanoic (phloionolic) acid occurs in plant cutins (2,3) and cork (see ref. 1 for discussion), but trihydroxy acids have not previously been reported as constituents of plant seed oils. In 1964 phloionolic acid was identified as a component of apple peels (4), and the triacetate of 8,9,13-trihydroxydocosanoic acid has been isolated from a yeast culture liquor (5).

Chamaepeuce afra (Jacq.) DC. (a composite) seed oil gave an infrared (IR) spectrum that showed hydroxyl (3560 cm^{-1}) and acetate (1230 cm^{-1}) absorption. Triglycerides comprised exclusively of normal fatty acids were present only in trace amounts in this oil, as indicated by thin-layer chromatography (TLC); instead two large spots attributable to somewhat more polar materials were observed. When *C. afra* oil was transesterified with acidic methanol, the resulting ester mixture was solid at room temperature. About 20% of this methyl ester mixture failed to emerge from gas-liquid chromatographic columns. These observations led to the hypothesis that *C. afra* oil contained hydroxy acids. The isolation and identification of these acids are reported in this paper.

Two other oil samples, from *C. hispanica* DC. seed and from seed of an unidentified *Chamaepeuce* species, were investigated briefly. Although these related species were examined less completely than the *C. afra*, the results obtained are included in this report.

EXPERIMENTAL

Materials and Methods

Infrared spectra were determined with 1% solutions in chloroform or carbon disulfide on a Perkin-Elmer Infracord, Model 137. Nuclear magnetic resonance (NMR) spectra were determined with a Varian A-60 spectrometer with deuterochloroform as solvent and tetramethylsilane as the internal reference. A Cary Model 60 spectropolarimeter was used for the optical rotatory dispersion (ORD) studies, and a Fisher-Johns block was used to determine melting points (uncorrected).

The *C. afra* seed oil was analyzed with a 50 x 0.3 cm O.D. stainless steel gas-liquid chromatographic column, packed with 3% JXR on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories Inc.). JXR (a dimethylpolysiloxane gum) is an effective liquid phase for triglyceride analyses (6,7). An F&M Model 810 "Research Chromatograph" equipped with a hydrogen flame detector was used, and the column temperature was programmed from 200C at 4C/min. Helium was the carrier gas.

All methyl ester samples were analyzed by gas-liquid chromatography (GLC) on a 275 x 0.3 cm I.D. glass column packed with 3% LAC-2 R-446 polyester on 60–80 mesh Celite and on a similar column with 5% Apiezon L grease as the liquid phase. These two columns were run isothermally (202C for the polyester; 208C or 257C for the Apiezon L) with helium as the carrier gas. Burrell Kromo-Tog K-5 chromatographs, equipped with thermal conductivity detectors, were used. Identification of component peaks (on GLC) was based on their equivalent chain-

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[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

lengths (8) as compared with equivalent chain-lengths of similar known materials. All percentages reported are area percentages.

Thin-layer chromatographic analyses of the trihydroxy methyl esters were done on 250 μ layers of Silica Gel G, silver nitrate-impregnated Silica Gel G (9), and Silica Gel G impregnated with boric acid (10). Petroleum ether (bp 30–60C)/diethyl ether (70/30) was the solvent for plain Silica Gel G plates whereas chloroform/methanol (98/2) was used for separations on the boric acid-impregnated plates. After various solvents were tested with the silver nitrate-impregnated plates, chloroform/methanol (96/4) was selected as giving the most satisfactory results. Visualization of spots was accomplished by charring the plates at 120C after they had been sprayed with a 50% aqueous H_2SO_4 solution of chromium trioxide.

Isolation and Analysis of Trihydroxy Acid Methyl Esters

Oil was extracted overnight from ground *Chamaepeuce afra* seeds with petroleum ether (bp 30–60C) in a Soxhlet extractor. An overnight Soxhlet extraction of the residual meal with diethyl ether yielded no additional lipid material. The mixed methyl esters were prepared by refluxing the oil for 3 hr with 1% H_2SO_4 in methanol.

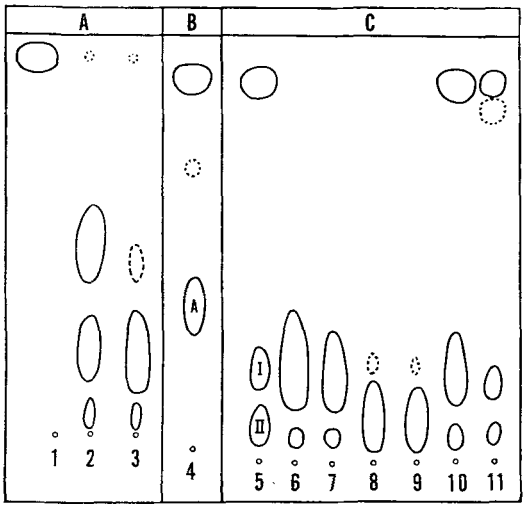

FIG. 1. Drawings of thin-layer chromatograms on: *A*, Silica Gel G developed with diethyl ether/petroleum ether (30/70); *B*, Silica Gel G impregnated with boric acid developed with methanol/chloroform (2/98); and *C*, Silica Gel G impregnated with silver nitrate developed with methanol/chloroform (4/96).

Samples are: *1*, soybean oil; *2*, *Chamaepeuce afra* oil; *3*, *C. hispanica* oil; *4* and *5*, *C. afra* methyl esters; *6*, *7*, *8*, and *9*, trihydroxy methyl ester fractions A, B, C, and D (from *C.* afra); *10*, *C. hispanica* methyl esters; and *11*, *Chamaepeuce* (unidentified species) methyl esters.

Crystallization of these mixed methyl esters, first from hot benzene (fraction A) at room temperature and then from ethyl ether (fraction B) at –18C, yielded essentially all the saturated trihydroxy methyl ester (I) in quite pure form as shown by TLC on Silica Gel G impregnated with silver nitrate. After the liquor residue from these crystallizations was recovered and dissolved in a large volume of ethyl ether/hexane (2/1), the solution was cooled at –18C for 2 days. The crystals that separated (fraction C) melted near room temperature and were nearly pure unsaturated trihydroxy methyl ester (II). Although TLC indicated that a considerable amount of trihydroxy material remained in the liquor fraction, further attempts to recover it by crystallization failed. Column chromatography was therefore applied to this material left in the liquor fraction. For this purpose a 1.4 x 25 cm column of Adsorbosil CAB (100–140 mesh, Applied Science Laboratories Inc.) was used with chloroform as the solvent. Fractions of 15 ml each were collected until nearly all the non-hydroxylated methyl esters were off the column. After the eluting solvent was changed to chloroform/methanol (96/4), the unsaturated trihydroxy methyl ester (nearly pure) was collected (fraction D). Monitoring the collected fractions was accomplished by TLC with plates of silver nitrate-impregnated Silica Gel G.

Oxidative cleavage of these trihydroxy methyl esters with permanganate-periodate and recovery of the products were done according to Von Rudloff's method (11). The resulting free acids were esterified with refluxing H_2SO_4 in methanol and analyzed by GLC.

Hydrogen uptake by the unsaturated trihydroxy methyl ester was measured in absolute ethanol with platinum oxide catalyst. After the saturated product (III) of this hydrogenation reaction was isolated, it was subjected to the bromination-debromination technique of Ames and Bowman (12) to determine the configuration of the vicinal dihydroxy grouping. This reaction was also applied to the saturated trihydroxy compound (I) that was isolated from the methyl ester mixture.

RESULTS AND DISCUSSION

Preliminary Analyses of Oils and Methyl Esters

When extracted with petroleum ether, ground *Chamaepeuce afra* (Jacq.) DC. seeds

yielded 24.4% of oil. Analysis of this oil by GLC and TLC (Fig. 1A, sample 2) indicated that triglycerides comprised exclusively of normal fatty acids were present only in trace amounts. Instead of a spot in the normal triglyceride region (compare with soybean oil, sample 1), two large spots and a smaller spot, all quite polar, were observed on the thin-layer chromatogram of *C. afra* oil. This pattern of spots was also observed on the chromatogram (not pictured) of the seed oil from the unidentified *Chamaepeuce* species. *C. hispanica* DC. seed oil gave a slightly different pattern (Fig. 1A, sample 3) from the other two oils; it showed only one major spot and two smaller ones. Probably the large polar spots are attributable to glycerides containing hydroxy acids. Acetate (1230 cm^{-1}) and hydroxyl (3560 cm^{-1}) absorption in the IR spectra of all three oils indicates that some of the hydroxyl groups are acetylated and some are free. *C. afra* oil is optically active, having $[\alpha]_D^{26°} + 2.1°$ (c = 12.7, $CHCl_3$), which is probably caused by the activity of the trihydroxy acid moieties. An investigation into the glyceride structure of these oils will be made and the results reported later.

After *C. afra* seed oil had been transesterified, the resulting mixed methyl esters were solid at room temperature and showed strong hydroxyl absorption in the IR. Analysis of these esters by GLC gave the following composition (excluding methyl acetate and trihydroxy esters): $C_{10:0}$, 2.3%; $C_{12:0}$, 8.1%; $C_{14:0}$, 0.5%; $C_{16:0}$, 10.9%; $C_{18:0}$, 1.4%; $C_{18:1}$, 15.8%; and $C_{18:2}$, 60.9%. A comparison between the total area of these peaks and the area obtained for the same amount of soybean oil methyl esters indicated that about 20% of the *C. afra* esters did not emerge from the columns.

A small sample of the trihydroxy compounds was crystallized from the ester mixture and was shown by a spot test on filter paper to consume periodate as rapidly as a sample of methyl 9,10-dihydroxystearate. This behavior led to the conclusion that vicinal diol groupings were present.

As shown in Fig. 1B, sample 4, the trihydroxy methyl esters of *C. afra* migrated as a single spot (spot A) on boric acid-impregnated Silica Gel G, indicating the presence of only one configurational isomer. The thin-layer chromatogram on Silica Gel G impregnated with silver nitrate (Fig. 1C) shows that the mixed methyl esters of all three of these oils (samples 5, 10, and 11) contain two trihydroxy compounds. Their migration pattern indicates that the upper spot is a saturated ester (I) and the lower spot is a monounsaturated ester (II). Sample 10, *C. hispanica* methyl esters, appears to have much more saturated than unsaturated trihydroxy ester, but no isolation work was done on this mixture.

Isolation and Identification of Trihydroxy Compounds from *C. afra* Seed Oil

The primary isolation and identification work was done on the mixed methyl esters from *C. afra* seed oil. A 4.78-g sample of these esters yielded 0.446 g of I and 0.684 g of II. Each of the four fractions (A, B, C, and D) isolated were analyzed by TLC on Silica Gel G impregnated with silver nitrate (Fig. 1C). Samples 6 and 7 (fractions A and B) are nearly pure I, mp 77–79C and samples 8 and 9 (fractions C and D) are slightly impure II, which melted near room temperature. All four of these fractions gave an IR free hydroxyl band (approximately 3710 cm^{-1}, $CHCl_3$) equal in intensity to that exhibited by methyl 9,10,16-trihydroxypalmitate. An additional band attributable to intramolecularly bonded hydroxyl groupings was observed as a shoulder at about 3620 cm^{-1}.

Methyl ester I (fractions A and B) was recrystallized from ethyl acetate to a constant mp of 80–81C [lit. mp of methyl phloionolate, 80.0–80.4C (2)]. The ORD spectrum of I (c = 2.3, absolute ethanol) was a plain positive curve with the following characteristics (at 25C): $[\alpha]_D + 22.3°$, $[\alpha]_{500} + 31.9°$, $[\alpha]_{400} + 51.4°$, and $[\alpha]_{300} + 104°$. As far as is known, the rotation of phloionolic acid (or methyl phloionolate) has not previously been reported, but the observed value agrees well with that obtained by Hopkins and Chisholm (13) for *threo*-12,13-dihydroxystearic acid. The conclusion to be drawn is that I has a *threo* configuration since *erythro* compounds generally have much lower rotations (14). This conclusion agrees with the results of other workers, indicating that phloionolic acid from other sources is the *threo* isomer (2,15).

Cleavage of I with permanganate-periodate yielded only nonanedioic (60%) and ω-hydroxynonanoic (35%) acids (by GLC of their methyl esters). Essentially the same ratio of dicarboxylic acid to ω-hydroxy acid (60/35) was found after a similar cleavage of methyl 9,10,16-trihydroxypalmitate.

The acid from the saponification of I had mp 102–104C, in agreement with a literature value of 102–103C (2). Found: C, 64.6; H, 10.83; neutral equivalent, 333. Calc. for

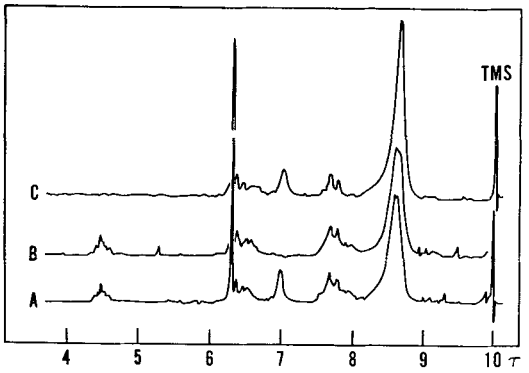

FIG. 2. Nuclear magnetic resonance spectra (60 mc) of: *A*, methyl *threo*-9,10,18-trihydroxy-*cis*-12-octadecenoate (II); *B*, II after exchange with D_2O; and *C*, methyl *threo*-9,10,18-trihydroxyoctadecanoate (I). (Both compounds are from *C. afra* oil.)

$C_{18}H_{36}O_5$: C, 65.02; H, 10.91; neutral equivalent, 332). Subjecting this acid to the bromination-debromination technique of Ames and Bowman (12) produced a monoenoic ethyl ester that gave only one spot on silver nitrate TLC, and that analyzed 86% isolated *trans* unsaturation by quantitative IR measurement (16). Analysis of this ester by GLC revealed 90% of C_{18} monoene, and 10% of materials with shorter retention times. The retention time of ethyl elaidate on this GLC column was 9.0 min; but the analyses of these monoenes resulting from the debromination reactions were continued for 90 min, which is longer than necessary to detect any residual monobromo elaidate (17). None was detected in any of the analyses, indicating that the debromination reaction (a large excess of zinc was used) effected hydrogenolysis of the 18-bromo group as well as removal of the 9,10-dibromo grouping. Other workers however, using slightly different conditions, have obtained 16-bromo-9-hexadecenoic acid (39%) by debromination of 9,10,16-tribromopalmitic acid (18). They did not identify the other products formed.

The NMR spectrum of I (Fig. 2C) indicated a saturated, terminal hydroxyl structure because signals caused by terminal C-methyl protons (*ca.* 9.1 τ) and to olefinic protons (*ca.* 4.6 τ) were not present. Disappearance of only the broad singlet at 7.0 τ after deuterium exchange is evidence that all three hydroxyl proton signals are incorporated in this band. Results prove conclusively that compound I is the (+)-*threo* isomer of methyl 9,10,18-trihydroxyoctadecanoate.

After recrystallization from ethyl acetate, compound II (fractions C and D) was a poorly crystalline substance with mp 33-35C. It had no absorption in the UV above 200 mμ and had no *trans* unsaturation according to IR. It consumed 1.1 moles of hydrogen per mole of ester with platinum oxide catalyst and absolute ethanol as solvent. A plain positive ORD curve (c = 2.6, absolute ethanol) was obtained for II, which had the following characteristics (at 25C): $[\alpha]_D$ + 18.2°, $[\alpha]_{500}$ + 26.2°, $[\alpha]_{400}$ + 43.4°, and $[\alpha]_{300}$ + 98.2°. Since this $[\alpha]_D$ agrees with a literature value of +18.9° (13) for an unsaturated *threo* diol, II also probably has the *threo* configuration.

The oxidative cleavage fragments from II were converted to methyl esters, and the esters were comprised of methyl nonanedioate and methyl ω-hydroxyhexanoate as shown by GLC. Saponification of II yielded the parent compound 9,10,18-trihydroxy-*cis*-12-octadecenoic acid, mp 76-76.5C.

The saturated trihydroxy methyl ester (III) resulting from hydrogenation of II had a mp of 78-79C, mixed mp with I, 77-79C and $[\alpha]_D^{22°}$ + 17.8° (c = 1.9, $CHCl_3$, visual polarimeter). Permanganate-periodate cleavage and analysis of III by TLC on silver nitrate-impregnated Silica Gel G also indicated that it was identical with compound I. When subjected to the bromination-debromination reaction, the saturated trihydroxy acid (mp 101-103C) derived from saponification of III yielded a monoene that assayed 96% isolated *trans* unsaturation by IR (16), gave one spot corresponding to a *trans* monoene on silver nitrate TLC, and was pure C_{18} monoene by GLC (analyzed as described for the debromination product from I). The results of this sequence of reactions indicate that the vicinal diol grouping in the unsaturated trihydroxy compound (II) had the *threo* configuration. Terminal C-methyl proton signals were not observed in the NMR spectrum of II (Fig. 2A), but the existence of a double bond was confirmed by an olefinic proton signal at 4.5 τ equivalent to two protons. The NMR spectrum that results from analysis of deuterium-exchanged II (Fig. 2B) shows that all the hydroxyl proton signals originally appeared at 7.0 τ in Fig. 2A. These data establish that II is the previously unknown (+)-methyl *threo*-9, 10, 18-trihydroxy-*cis*-12-octadecenoate.

Trihydroxy Compounds from Unidentified *Chamaepeuce* Seed Oil

Isolation of the trihydroxy methyl esters from the unidentified *Chamaepeuce* seed oil was accomplished in the manner described for *C. afra*. The total amount of trihydroxy ester present in the mixture was about 18%. One fraction (0.009 g) had a mp of 79.5-80.0C [lit. mp for methyl phloionolate, 80.0-80.4C (2)]. The intensity of the IR hydroxyl band was equivalent to that of pure methyl 9, 10, 16-trihydroxypalmitate. Permanganate-periodate cleavage of 0.006 g of this purified trihydroxy ester yielded only nonanedioic (61%) and ω-hydroxynonanoic (36%) acids (by GLC of their methyl esters). These results indicate that the trihydroxy compound from this unidentified *Chamaepeuce* seed oil is methyl 9, 10, 18-trihydroxyoctadecanoate. Some unsaturated trihydroxy material was also shown to be present (Fig. 1C, sample 11), but it was not isolated. The configuration of the 9, 10-diol grouping in these trihydroxy acids is not known because the amount of seed oil available was insufficient to obtain conclusive evidence.

ACKNOWLEDGMENT

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The Monoene and Other Wax Alcohols of Human Skin Surface Lipid and Their Relation to the Fatty Acids of This Lipid[1,2]

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ABSTRACT

1) Wax alcohols (as acetates) were isolated from human skin surface lipid and separated into a saturated and a monoene fraction.

2) Four main chain types were found for both saturated and monoene alcohols: normal even, normal odd, iso and anteiso. ("Even" and "odd" refer to the number of C-atoms in the straight chain.)

3) The monoene alcohol acetates were separated into homologues of each chain type by preparative gas-liquid chromatography (GLC) and the positions of the double bonds for each homologue were determined by analytical GLC of the original fraction, its hydrogenated derivative, and the products it formed by reductive ozonolysis.

4) The fragments formed by reductive ozonolysis of the monoene alcohol acetates were compared to those formed from the total monoenoic fatty acids (as methyl esters), both obtained from the same sample of surface lipid. (Comparisons were best made by ozonolysis of a portion of the entire sample of each ester group.)

a) The terminal ends of both groups of monoene fatty chains yielded a very similar pattern of aldehydes in terms of types and amounts. This could be explained by the hypothesis that both fatty acid and fatty alcohol chains of lengths ranging mainly from C_{14} to C_{18} were first biosynthesized, then desaturated at $\Delta 6$.

b) The functional group ends gave a distinct pattern of aldehyde esters for the acids and another for the alcohols. Both patterns consisted nearly entirely of members having an *even* number of C-atoms from the double bond to the functional group. This suggested that the members of each pattern were formed by chain extensions of an integral number of C_2 units beyond the lengths arrived at in 4a). Thus 71% of the fatty acid monoenes were not extended, 25% were extended by 1 C_2 unit and the remainder extended from 2 to 5 C_2 units, whereas nearly all the fatty alcohols were extended mainly by 2, 3 or 4 C_2 units, with decreasing amounts up to 8 C_2 units.

5) A small amount ($\sim$5%) of odd chain aldehyde esters for both fatty acids and fatty alcohols were found and some unidentified alcohols were detected.

INTRODUCTION

WAX ESTERS, which make up 20 to 25% of human skin surface lipid, consist of fatty alcohols esterified to fatty acids. The complex nature of the alcoholic portion of these esters was originally revealed by mass spectrometry (1). This technique showed the presence of a saturated and an unsaturated series each ranging from C_{18} to C_{27}, with chain lengths of odd as well as even numbers of C-atoms present, the C_{20} chain being most abundant.

Subsequently, Hougen isolated and identified 12 alcohols from this source: six saturated alcohols of even chain lengths from C_{14} to C_{24}, three saturated *iso* alcohols from C_{20} to C_{24}, and eicos-10-en-1-ol, docos-12-en-1-ol and tetracos-14-en-1-ol, all with *cis* double bonds (2).

Two gas-chromatographic studies (3,4) revealed the presence of additional homologues and unidentified branched chain compounds, both saturated and unsaturated.

From fragmentary earlier data it was postulated (5) that in the sebaceous gland common mechanisms exist for building up and desaturating the fatty chains, some of which form the fatty acids of the various esters of sebum, and some the fatty alcohols. The present study, together with two studies (6,7) on the positions of the double bonds in the fatty acids of

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[1]Presented at the AOCS Meeting in Los Angeles, April 1966.

[2]Special terms and abbreviations:
Normal even = a straight chain with an even number of carbon atoms.
Normal odd = a straight chain with an odd number of carbon atoms.
i = iso = a straight chain with a methyl group at the ω –1 position.
a = anteiso = a straight chain with a methyl group at the ω –2 position.

surface lipid provide further insight into this process.

EXPERIMENTAL

The preparation and work-up of the fatty alcohols and fatty acids studied in this paper are diagrammatically summarized in Fig. 1.

A sample of human skin surface lipid (3.720 g) was collected from a young man, age 27, from 10 scalp soakings (8), then stored in a sealed tube. A 0.752 g portion of this sample was chromatographed on Mallinckrodt silicic acid which had previously been washed and deoxygenated (9), then stored under $CHCl_3$. The adsorbent was packed into a chromatographic column 4.5 cm I.D. to a height of 17 cm and washed with about 600 ml of $CHCl_3$, whereupon a 30-ml sample of effluent gave a negligible residue. The $CHCl_3$ was washed off the column with 500 ml of hexane, and the sample in 10 ml hexane was applied to the column and washed into it with five 2-ml washes of hexane. The flow rate was 10 ml/min and tubes each containing 40 ml of eluate were collected. Eluting solvents were hexane followed by increasing concentrations of benzene in hexane, increasing concentrations of diethyl ether in benzene, diethyl ether and finally methyl alcohol. (All solvents in this study were redistilled, the diethyl ether being redistilled just before use. To the chloroform 0.5% methanol was added as a preservative.) Aliquots were taken of each tube for TLC, which was performed as previously described (10). For some fractions the Liebermann-Burchard test was also run.

For the examination of the wax alcohols a pooling was made of an aliquot (two-thirds) of the contents of all tubes which showed one spot on thin-layer chromatography (TLC) (10) in the region where wax esters plus sterol esters migrate in hexane/ether 95/5. This yielded 119.5 mg which corresponded to a yield of 179.3 mg for the entire surface lipid sample. It was estimated that the pooled material represented 99% of the eluate of wax ester plus sterol ester. (The remaining 1%, being contaminated with products eluted just before and just after the bulk of this fraction, was not included in the pool.) The esters were refluxed with 10 ml 10% KOH in 90/10 ethanol/water for 2 hr with nitrogen bubbling slowly through the saponification mixture and the fatty acids (54.8 mg) separated from the nonsaponifiable matter (69.1 mg) in two stages as previously described (8), (recovery 100%). An aliquot of the nonsaponifiable matter was removed for TLC, which now showed no spot where the original wax ester and sterol ester migrated, but intense spots where fatty alcohols and cholesterol migrated. The remaining 65.2 mg was acetylated with a mixture of acetic anhydride and acetic acid, 4/1 (2 hr reflux with nitrogen bubbling through the reaction mixture). The excess reagents were blown off with nitrogen. TLC showed nothing in the region where free alcohols or free cholesterol migrated when 400 μg of the acetylated product was applied to the plate. This indicated that the acetylation was complete.

TABLE I

Chromatography of the Acetates of the Wax Alcohols of Human Skin Surface Lipid on Silicic Acid Impregnated with Silver Nitrate

Tube No. (8 ml each)	Eluent	Wt, mg	Remarks
1–7	Hexane	0.64	Unidentified
8–11	10% benzene in hexane	0	
12–23	10% benzene in hexane	33.68	Saturated alcohol acetates[a] (gave a yellow-green LB test)
24–37	10% benzene in hexane	7.32	Cholesteryl acetate
38–54	10% benzene in hexane	0.87	Unidentified (monoene alcohol acetates?)
55–73	30% benzene in hexane	13.54	Monene alcohol acetates
74–93	50% benzene in hexane	0.41	Monoene alcohol acetates (?)
94–102	Benzene	1.70	Polyene alcohol acetates (?)
103–112	Ether	3.35	Unidentified
113–117	Methanol	1.35	Unidentified
	Total recovery	62.86 = 98%	

[a]This material gave 32.2 mg of a fraction which formed urea adduct and 1.47 mg of a fraction which did not. The latter gave an intense yellow-green color with the Liebermann-Burchard test whereas the former was negative.

The acetylated alcohols were then chromatographed on a column (1.65 cm I.D. × 12 cm) of silicic acid impregnated with $AgNO_3$ (Adsorbosil ADN-1, purchased from Applied Science, State College, Pa.) as previously described (6). The saturated alcohol acetates (tubes 12-23, Table I), were subjected to the urea adduct separation (6) whereupon 95% formed adduct. The portion of this fraction forming urea adducts (which consisted of straight, *iso* and *anteiso* chains) was analyzed by GLC. The portion not forming urea adducts (i.e., more highly branched compounds) was mainly sterol. The monoenes (tubes 55 to 73, Table I) were then separated into fractions of a single chain length and type by preparative GLC and the double bond positions of each fraction determined by analytical GLC of the original fraction, its saturated analogue,

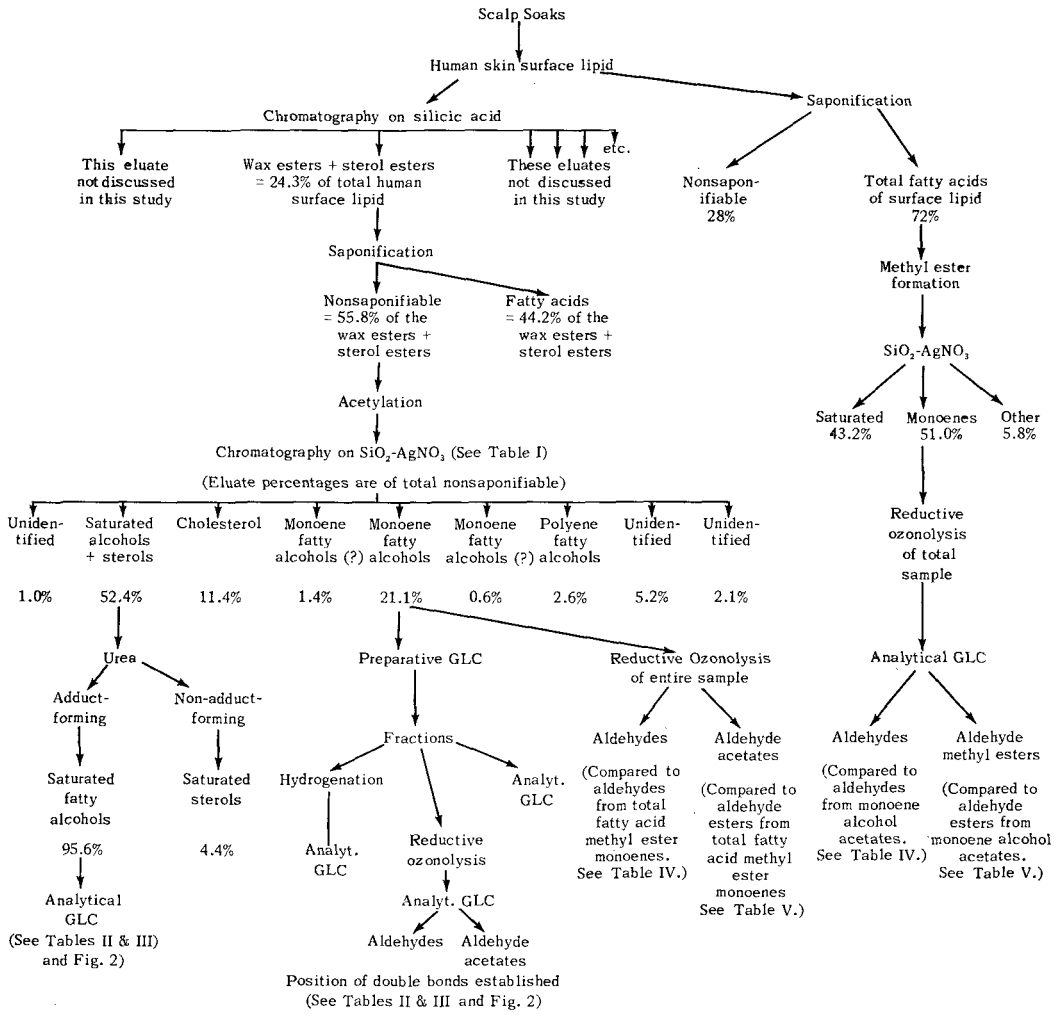

FIG. 1. Preparation and work-up of the fatty alcohol portion of the wax ester plus sterol ester fraction and the total fatty acid fraction of human skin surface lipid.

and products formed by reductive ozonolysis (6,7).

For comparison of the chains of the wax alcohols with those of the total fatty acids of this sample of surface lipid, a portion of the total unchromatographed surface lipid was saponified, the fatty acids recovered, methylated, and separated into a monoene fraction by procedures already described (6). The entire sample of monoenes of this sample was ozonized and analyzed by programmed GLC as described below.

Gas chromatography was performed on a Loe Model 160 instrument equipped with a hydrogen flame ionization detector. Preparative GLC was performed on an 8 ft × ¼ in. O.D. stainless steel column packed with 4% SE 30 on acid washed silanized Chromosorb G, 60/80 mesh. The column was programmed from 180C to 260C at the rate of 2C/min to 260C and isothermally thereafter. Helium, at a flow rate of 75 ml/min, was the carrier gas. A splitter sent one tenth the effluent to the detector and fractions appearing in the remaining nine tenths were trapped in capillary tubes. Analytical GLC of the collected fractions was performed on two columns; a) a 10 ft stainless steel column, ⅛ in. O.D. packed with 3% JXR on Chromosorb Q, and b) an 8 ft. stainless steel column, ⅛ in. O.D. packed with 8%

TABLE II

Proportion of Various Types of Saturated and Unsaturated Fatty Alcohols in Human Surface Lipid

Chain type	Fatty Alcohols Saturated	Monoenes
	%	%
Normal even	50	65
Normal odd	9	3
Iso	26	25
Anteiso	13	5
Other	2	2
	100	100

See footnote on p. 98 for definition of chain types.

EGSSX on Gas Chromosorb P, both packings prepared by Applied Science, Inc. Both isothermal and programmed analyses were performed, the latter being preferable for ozonolysis studies. Programs were from 80C to 190C at 4C/min and 30 ml/min He.

Standard alcohols with double bonds at different positions in the chain were prepared by $LiAlH_4$ reduction of a series of monoenoic elaidic acids (gifts of R. J. Meyer, Morton Salt Co., Woodstock, Ill.), where the double bonds were Δ10, Δ11, Δ12, Δ13, Δ14, and Δ16. Iso and anteiso alcohols (gifts from A. W. Weitkamp) were similarly prepared. Oleyl alcohol (Δ9) was purchased from the Hormel Institute, Austin, Minn. The standard alcohols were all acetylated by the same procedure described above.

Uncorrected areas as measured by triangulation or by peak height × width at half height were used and gave values approximately ±5% of standards.

The effects of our procedures (saponification, chromatography on SiO_2-$AgNO_3$, acetylation and methyl ester formation) were checked for double bond migration by comparison of the ozonolysis products formed by appropriate samples before and after resubjecting them to the same procedure. In no case did we find alteration of the pattern of products formed greater than 0.5%.

RESULTS

Figure 1 presents the yields of the fractions obtained at various stages of the work-up. The total proportion of the different chain types of saturated and unsaturated alcohols are given in Table II. Evidence for the assignment of these structures to the various chain types is discussed below. Figure 2 gives the homologue distribution of the saturated and monoene wax alcohol acetates. Table III gives the relative amounts of the individual molecular

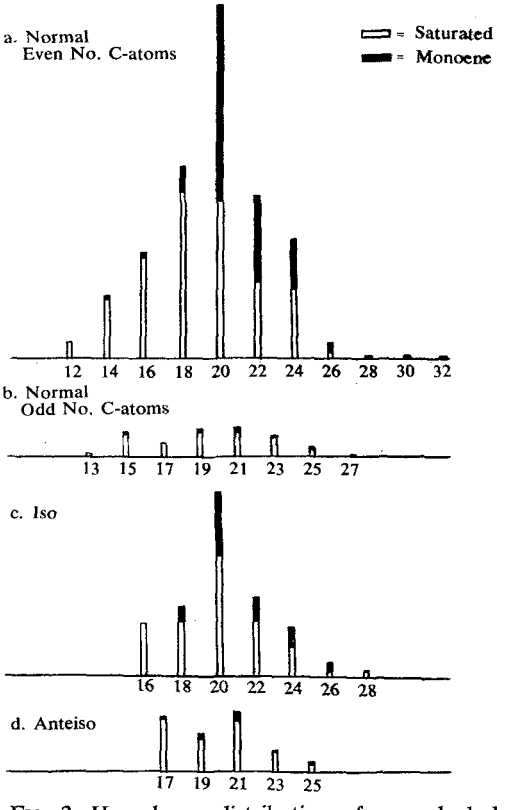

FIG. 2. Homologue distribution of wax alcohol acetates.

species for each chain type of unsaturated alcohol. Although trace amounts of monoenes were found for chain lengths up to C_{32}, the saturated alcohols were not examined above C_{27} since mass spectrometry (1) did not reveal any significant amount of material (either saturated or unsaturated) above this chain length. Table III also gives the results of the ozonolysis studies of the monoene alcohol acetates with position assignments of the double bonds. Besides the homologues of the chain types listed therein, ozonolysis revealed the presence of other types of branching. This became especially apparent when small amounts of material were collected, i.e., in the low and in the high molecular weight ranges. Branching appeared to be present in some of the aldehyde ester fragments derived from monoenes of chain lengths from C_{26} to C_{28}. The nature of these minor components is under further study.

Table IV gives the relative yield of the various types of aldehydes formed when a portion of the total sample of monoene fatty alcohol acetates are reductively ozonized. These aldehydes are compared to those formed from

TABLE III

Position Isomers at Each Chain Length of the Acetates of the Monoene Wax Alcohols of Human Skin Surface Lipid

Experimental data				Deduced structure								
		Ozonolysis products		Double bond position isomers and relative amount								
Monoene alcohol ECL after hydrogenation	Amount (% total monoenes)	Aldehyde acetate ECL	Aldehyde ECL	Normal even	%	Normal odd	%	Iso	%	Anteiso	%	Possible mode of isomer formation by C_2 extension of Δ6 chains
14.00	0.4											
15.00	0.4											
15.65	0.6											
16.00	0.7	6	10	16:Δ6	60							not extended
		8	8	16:Δ8	5							14:Δ6 + C_2
16.62	0.03											
17.00	0.07	6	11			17:Δ6	60					not extended
		8	9			17:Δ8	40					15:Δ6 + C_2
17.60J	2.3	6	11.6					i-18:Δ6	8			not extended
17.45E		8	9.6					i-18:Δ8	92			i-16:Δ6 + C_2
		6	12.0	18:Δ6	1							not extended
18.00	4.1	8	10.0	18:Δ8	76							16:Δ6 + C_2
		10	8.0	18:Δ10	23							14:Δ6 + 2 C^2
18.30	0.6											
18.70J	1.1	8	10.7							a-19:Δ8	82	a-17:Δ6 + C_2
		10	8.7							a-19:Δ10	18	a-15:Δ6 + 2 C_2
19.00	1.3	8	11.0			19:Δ8	13					17:Δ6 + C_2
		10	9.0			19:Δ10	87					15:Δ6 + 2 C_2
		8	11.6					i-20:Δ8	5			i-18:Δ6 + C_2
19.60J	11.5	10	9.6					i-20:Δ10	95			i-16:Δ6 + 2 C_2
		12	7.6					i-20:Δ12	tr			i-14:Δ6 + 3 C_2
		8	12.0	20:Δ8	2							18:Δ6 + C_2
20.00	29.1	10	10.0	20:Δ10	85							16:Δ6 + 2 C_2
		12	8.0	20:Δ12	13							14:Δ6 + 3 C_2
20.22	0.9											
		8	12.65							a-21:Δ8	4	a-19:Δ6 + C_2
20.74J	2.5	10	10.70							a-21:Δ10	94	a-17:Δ6 + 2 C_2
		12	8.70							a-21:Δ12	2	a-15:Δ6 + 3 C_2
21.00	1.7	10	11.0			21:Δ10	44					17:Δ6 + 2 C_2
		12	9.0			21:Δ12	56					15:Δ6 + 3 C_2
21.61J	4.9	10	11.6					i-22:Δ10	22			i-18:Δ6 + 2 C_2
21.52E		12	9.6					i-22:Δ12	78			i-16:Δ6 + 3 C_2
		10	12.0	22:Δ10	6							18:Δ6 + 2 C_2
22.00	15.2	12	10.0	22:Δ12	71							16:Δ6 + 3 C_2
		14	8.0	22:Δ14	23							14:Δ6 + 4 C_2

22.35	0.8										
		10	12.71						a-23:Δ10	13	a-19:Δ6 + 2 C_2
22.72	0.7	12	10.71						a-23:Δ12	75	a-17:Δ6 + 3 C_2
		14	8.71						a-23:Δ14	12	a-15:Δ6 + 4 C_2
		10	13.0		23:Δ10	7					19:Δ6 + 2 C_2
23.00	1.1	12	11.0		23:Δ12	66					17:Δ6 + 3 C_2
		14	9.0		23:Δ14	27					15:Δ6 + 4 C_2
		10	13.6				i-24:Δ10	2			i-20:Δ6 + 2 C_2
23.62J 23.52E	3.8	12	11.6				i-24:Δ12	12			i-18:Δ6 + 3 C_2
		14	9.6				i-24:Δ14	86			i-16:Δ6 + 4 C_2
		10	14.0	24:Δ10	4						20:Δ6 + 2 C_2
24.00	10.5	12	12.0	24:Δ12	3						18:Δ6 + 3 C_2
		14	10.0	24:Δ14	83						16:Δ6 + 4 C_2
		16	8.0	24:Δ16	10						14:Δ6 + 5 C_2
24.75J	0.5	14	10.7						a-25:Δ14	maj	a-17:Δ6 + 4 C_2
		14	11.0		25:Δ14	56					17:Δ6 + 4 C_2
25.00	0.5	16	9.0		25:Δ16	44					15:Δ6 + 5 C_2
		14	11.65				i-26:Δ14	13			i-18:Δ6 + 4 C_2
25.62J	1.6	16	9.61				i-26:Δ16	87			i-16:Δ6 + 5 C_2
		14	12.0	26:Δ14							18:Δ6 + 4 C_2
26.00	1.5	16	10.0	26:Δ16	maj						16:Δ6 + 5 C_2
		18	8.0	26:Δ18							14:Δ6 + 6 C_2
26.73J	tr	16	10.71						a-27:Δ16	maj	a-17:Δ6 + 5 C_2
27.00	0.2	16	11		27:Δ16	maj?					17:Δ6 + 5 C_2
		16	11.58				i-28:Δ16				i-18:Δ6 + 5 C_2
27.60J	0.3	18	9.61				i-28:Δ18	maj			i-16:Δ6 + 6 C_2
		20	?				i-28:Δ20				i-14:Δ6 + 7 C_2
28.00	0.7	18	10.0	28:Δ18	maj						16:Δ6 + 6 C_2
		20	?	28:Δ20							14:Δ6 + 7 C_2
28.7	0.2										
29.0	0.2										
29.6	tr										
30.00	0.1	18	?	30:Δ18	5						18:Δ6 + 6 C_2
		20	10.0	30:Δ20	95						16:Δ6 + 7 C_2
30.7	tr										
31.0	tr										
31.6	tr										
32.00	tr	20	?	32:Δ20	5						18:Δ6 + 7 C_2
		22	10.0	32:Δ22	95						16:Δ6 + 8 C_2

ECL, equivalent chain length or carbon number as defined by Woodford & Van Ghent (11) (a fractional ECL generally means branching and/or unsaturation); J, stationary phase JXR; E, stationary phase EGSSX; i-, iso; a-, anteiso; maj, major position isomer; tr, trace.

TABLE IV

Relative Amounts of Aldehydes of the Main Chain Types Released from Ozonolysis of the Total Monoenoic Acids and from the Total Monoene Wax Alcohols of Human Surface Lipid[a]

Chain types:	Normal even		Normal odd		Iso		Anteiso	
	Aldehyde released (ECL)	% of Total aldehydes	Aldehyde released (ECL)	% of Total aldehydes	Aldehyde released (ECL)	% of Total aldehydes	Aldehyde released (ECL)	% of Total aldehydes
Total fatty acid monoenes	8	7.8						
			9	6.6			8.7	1.6
	10	64.0			9.6	8.0		
			11	4.8			10.7	1.6
	12	2.7			11.6	2.7		
							12.7	trace
		74.5		11.4		10.7		3.2
Total wax alcohol monoenes	8	4.6						
			9	3.0			8.7	trace
	10	65.6			9.6	12.0		
			11	1.8			10.7	3.6
	12	4.1			11.6	4.8		
							12.7	.5
		74.3		4.8		16.8		4.1

[a] Abbreviations as in Table III and footnote, p. 98.

the total monoenoic fatty acid methyl esters. Table V gives the yields of aldehyde esters formed from the monoene alcohol acetates and fatty acid methyl esters when the total samples are reductively ozonized.

DISCUSSION

From the data of Fig. 1 it can be computed that the monoenoic fatty chains (i.e., fatty acids and fatty alcohols) that make up human skin surface lipid are about 40% of the lipid whereas the saturated chains constitute about 38%. Thus, in terms of quantity at least, these chains constitute the bulk of surface lipid which is primarily the product of the sebaceous glands (sebum). Their manner of formation is therefore of some interest to cutaneous biology in particular and perhaps to lipid chemistry in general.

It was earlier postulated (5) that in the sebaceous glands common mechanisms exist for building up and desaturating the hydrocarbon chains that eventually become the fatty acids and the fatty alcohols of the various esters of sebum. It was further postulated that the chains which were to become fatty alcohols were extended on the average of two C_2 units more than the chains which became fatty acids. These conclusions were reached from a) a comparison of the average number of double bonds per molecule of the wax alcohols and free fatty acids of human surface lipid (8), b) a comparison of the double bond position of the free fatty acids (12) with those of the alcohols (2), and c) a comparison of the

TABLE V

Mole % Aldehyde Esters Formed from the Monoenes of the Total Wax Alcohol Acetates and the Total Fatty Acids

Aldehyde ester	No. of C_2 units chain extended above C_6	Mole % aldehyde acetates		Mole % aldehyde methyl esters from gas chrom. of total fatty acid monoenes
		Computed from gas chrom. data of total wax alcohol monoenes	Computed from position isomer data of Table III	
C_6	0	1.4	0.6	71.1
C_8	1	7.6	9.0	25.3
C_{10}	2	45.9	48.7	3.0
C_{12}	3	24.3	22.6	0.6
C_{14}	4	15.5	16.5	trace
C_{16}	5	4.2	2.5	trace
C_{18}	6	0.7	trace	
C_{20}	7	0.2	trace	
C_{22}	8	0.2	trace	

chain types of fatty acids and fatty alcohols as they were known at that time. Later it was found that for the fatty acid monoenes of the four main types of carbon skeletons of surface lipid (i.e., normal even, normal odd, iso and anteiso) the double bond position was either at Δ6 or could be derived from this position by chain extension at the carboxyl group by an integral number of C_2 units (7,10,13). The data of this paper support this hypothesis that the fatty chains, which make up all the acids of the various lipid classes and all the fatty alcohols of the wax esters have a common origin and a common desaturation mechanism. It also gives some details as to chain extension processes which can account for the chain lengths actually found for the alcohols and the acids.

The processes envisioned here can be summarized as follows (Fig. 3):

1) Four main types of fatty chains (normal even, normal odd, iso and anteiso) are first biosynthesized to lengths mainly from C_{14} to C_{18} with C_{16} in by far the greatest amount. The chains at this stage are presumably as acyl CoA derivatives.

2) Some of these chains are then desaturated to form Δ6 monoenes. (Small amounts of polyenes are also formed. These have not been studied in this paper.)

3) The Δ6 monoene chains are then extended at the functional group side of the molecule by C_2 units according to two distinct chain extension patterns. The first of these forms the chains which become the monoenoic fatty acid moieties of the various esters of sebum, and the second the monoene fatty alcohol moieties of the wax esters. It is probable that the saturated chains are also extended by the same chain extension patterns as those that form the unsaturated fatty acids and fatty alcohols.

4) A small amount (~5%) of the chains formed do not fit the above scheme. Such chains could conceivably arise by extension of a Δ7 or Δ9 chain by C_2 units or by decarboxylation of the normal Δ6 or the Δ6 plus C_2 extended chain.

Each of these processes will be discussed in turn:

1) *The Chain Types of Sebum.* The structures and biosynthesis of the various fatty acid chain types found in surface lipid has been reviewed (6,13). Establishment of the structures of the odd and even fatty alcohols was done by mass spectrometry (1).

Assignment of the structures *iso* and *anteiso*

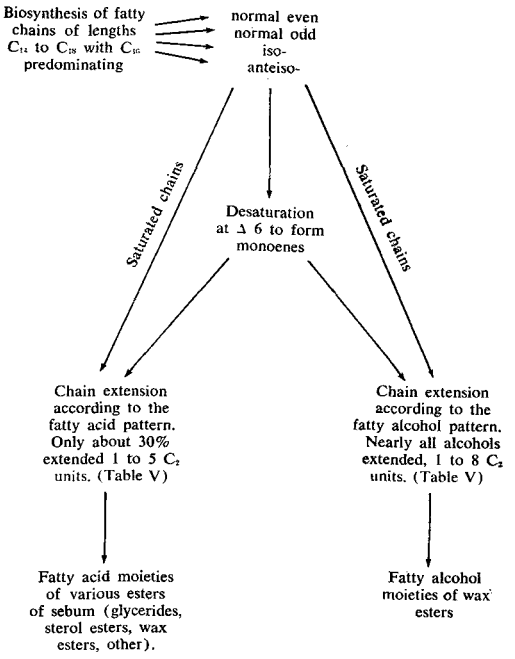

FIG. 3. Scheme for biosynthesis of the bulk of the fatty acids and fatty alcohols that occur in sebum.

to the fatty alcohols is based upon the following considerations: a) Hougen isolated 3 alcohols whose melting points and infrared spectra corresponded to C_{20}, C_{22} and C_{24} iso alcohols (2). b) We found that when the monoene alcohol acetates are cleaved by ozonolysis the aldehyde rather than the aldehyde ester fragment shows the branching. The fractional carbon number (11) or equivalent chain length (ECL) for an iso or anteiso aldehyde is the same as that of a methyl ester, i.e., 0.60 for iso and 0.72 for anteiso on JXR, a nonpolar liquid phase for GLC. c) The naturally occurring saturated or the hydrogenated monoene alcohols give the correct equivalent chain lengths (ECL) for the iso or anteiso structure (i.e., 0.60 for iso and 0.72 for anteiso on JXR). (One would not expect the fractional ECL of an iso or anteiso alcohol acetate to be very different from the corresponding methyl ester, and indeed, this is what standard substances show, but it is noteworthy that aldehydes also show the same ECL for these types of branching.) d) Finally, as will be discussed below, the same biosynthetic processes which can explain the build up of fatty acid chains, where mass spectrometry has shown both iso and anteiso structures to be present (6), can

also explain the structures of the fatty alcohol chains. Thus, although these considerations do not rigorously prove that all the homologues reported as iso or anteiso in this study are such, they do make these structures highly probable.

2) *The Formation of Δ6 Monoenes.* Reductive ozonolysis of a monoene fatty acid methyl ester or a fatty alcohol acetate yields an aldehyde and an aldehyde ester in each case. If the sequence of steps hypothesized above for the formation of the monoene chains of sebum is correct, then, on reductive ozonolysis, we would expect to obtain the same types and amounts of aldehydes from the fatty alcohol monoenes as we do from the fatty acid monoenes. In a sense the double bond becomes a natural label. A sample of the total fatty alcohol acetate monoenes and the total fatty acid methyl esters (both obtained from the same individual to avoid possible biological variations) was reductively ozonized and the pattern of aldehydes obtained in each case compared (Table IV). The similarity of aldehydes obtained from the two groups of monoenes is striking—a fact which strongly supports their common biosynthetic origin. The relatively small differences that do exist, as for example between the normal odd chains or the iso chains, may indicate that the acids or the alcohols favor certain chain types, i.e., iso chains preferentially form fatty alcohols rather than fatty acids, and normal odd chains preferentially form fatty acids rather than fatty alcohols. Normal odd chain aldehydes might also arise to a small degree from fatty chains of the more common Δ9 type, e.g., from oleic acid. The free fatty acids of surface lipid contain about 2% of this fatty acid which is thought to be derived from keratinizing epidermis or bacteria rather than from the sebaceous gland (7).

3) *Further Chain Extensions.* Since the same aldehydes are formed from ozonolysis of monoene chains either of fatty acids or of fatty alcohols in roughly the same amounts, differences in chain length of these types of compounds must be reflected in their aldehyde esters. If, as was assumed above, Δ6 monoenes are formed after chains mainly from C_{14} to C_{18} length are first built up, the amount of chain extension of a given monoene can be determined by noting how much longer is the chain length of the aldehyde ester in question compared to a C_6 aldehyde ester. Data for fatty alcohol acetate monoenes are given in Table III. Note that position isomers for any given chain type differ from each other by 2 C-atoms. This is also true for the fatty acids (7). These differences can be explained if various chain types with double bonds at Δ6 are extended an integral number of C_2 units (see last column of Table III).

It is possible to determine the total amount of each aldehyde acetate by ozonolysis of the total sample of alcohol acetate monoenes. It is also possible to compute the total amount of aldehyde acetate of each chain length for the *total* fatty alcohol sample from the data of Table III. The mole percent of each monoene is first computed from the weight percent, then, from the percent of each position isomer present for each monoene, the moles of aldehyde acetate of each chain length can be calculated, totaled and percentages computed. Results from such a calculation are presented in Table V and these are compared to the mole percent of the same aldehyde acetates that were determined by ozonolysis of the *total* sample of alcohol acetate monoenes. The rather close correspondence between the two independently determined sets of results shows the internal consistency of the data.

The aldehyde acetate pattern shows a maximum at C_{10} but extends all the way to C_{22}. If the chains are extended by C_2 units from a Δ6 chain starting point, as hypothesized here, this would mean that from 0 to 8 C_2 units and maximally 2 C_2 units are added. For comparison the chain extension pattern for the total fatty acids is also tabulated in Table V. Note that only about one fourth of these chains are extended above C_6 aldehyde methyl ester, where as with the fatty alcohols nearly all the chains are extended above C_6 aldehyde acetate.

Although the scheme proposed here has been revealed by the unusually occurring Δ6 double bond, which served as a natural label, it is very likely that saturated chains for both acids and alcohols could also be formed by similar mechanisms, i.e. first buildup of chains from C_{14} to C_{18} with C_{16} in greatest amount, then further extension of these chains according to either the fatty alcohol or the fatty acid chain extension patterns. This seems plausible since the same overall chain lengths which characterize either fatty alcohols or the fatty acid monoenes occur in the saturated series of compounds.

4) *Other Chain Formation Processes.* In addition to the aldehyde ester pattern of the fatty alcohols described above, which accounts for about 95% of the wax alcohols, small but definite amounts of *odd* chain aldehyde esters were found for chain lengths ranging from C_7

to at least C_{21}. The total fatty acids also showed small amounts of aldehyde methyl esters ranging from C_5 to C_{19}. That these odd-chain aldehyde esters are not primarily due to artifacts of procedure was ruled out by the fact that the ozonolysis procedure itself showed less than 1% over oxidation or double bond migration (14), and that none of our procedures (such as saponification, chromatography on silicic acid-silver nitrate, acetylation or methyl ester formation) altered the ozonolysis patterns more than 0.5% before and after resubjecting the sample to the same procedure.

Two possible ways in which fatty chains which would yield odd-chain aldehyde esters might be formed by known biochemical processes are: 1) $\Delta 9$ desaturation followed by chain buildup or degradation by C_2 units, or 2) decarboxylation of the normal $\Delta 6$ or the $\Delta 6$ plus C_2 extended chain (15,16). Which of these mechanisms, if either, is biologically operative to form the odd-chain aldehyde esters found in this study remains to be determined.

There is a possibility that some of the unsaturated acids such as $\Delta 8$, $\Delta 6$ and others which would yield an *even* chain aldehyde ester could arise by decarboxylation of a $\Delta 7$ monoene or the common $\Delta 9$ monoene chain followed by further C_2 degradation and/or C_2 chain extension. If the amount of odd-chain aldehyde ester formed is any indication of the amount of such decarboxylation, it must be small ($\sim 5\%$).

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SHORT COMMUNICATIONS

Fatty Acids of the Lipids from *Pullularia pullulans*

ALTHOUGH THE YEAST-LIKE FUNGUS, *Pullularia pullulans,* is one of the most common fungi in nature, the genus *Pullularia* has been little studied and taxonomic reports are rather confusing. In recent years a number of such organisms has been found in various parts of the human body thought to be inaccessible to bacteria, providing that the skin surface remains unbroken. Wynne and Gott (1) reported the presence of *P. pullulans* in a jugular lymph node of a patient with Hodgkin's granuloma, and yet another group noted the micro-organisms in rheumatoid arthritic joints.

In previous studies from this laboratory the growth requirements, pigment formation and isolation, and lipid composition of *P. pullulans* and related organisms were investigated. The present report presents gas chromatographic analysis of the fatty acids obtained on alkaline saponification of the lipids.

Strain NRRL YB–4515 of *P. pullulans* was grown in a medium containing the Difco preparations, 0.3% yeast extract, 0.3% malt extract, and 1.5% glucose in addition to 0.0125% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.2% NH_4Cl. Glucose was sterilized separately and added aseptically to the rest of the sterile medium. Flasks, each containing 20 milliliters of medium, were inoculated with *P. pullulans* and shaken mechanically for 24 hr at 27C. After three subsequent transfers, the last of which was into 12-liter flasks containing 6 liters of medium each, the cultures were centrifuged and washed first with 1% NaCl solution, then with triply distilled water until free from chloride and lyophilized.

Two samples of lyophilized cells (38.5 and 25.0 g) were shaken with chloroform-methanol (2:1, v/v, 50 ml/g) for 6 hr at 25C. The suspensions were centrifuged, and the cells were transferred to lipid-free thimbles and subjected to Soxhlet extraction with the same solvent mixture for 3 hr. The filtered extracts were combined and concentrated in a rotary evaporator at 30C.

For the saponification, the lipids were heated with 0.5% KOH in 95% ethanol. After removal of the unsaponifiable matter by ether, the residue was acidified and the free fatty acids were extracted with ether. Portions of the unsaponifiable matter were dissolved in ethanol and tested for the presence of carotenes and vitamin D_2.

For the gas chromatographic studies, esterification of the extracted fatty acids was accomplished by refluxing with boron trifluoride 10% in methanol for 30 min. The mixture was diluted with water, and the methyl esters were extracted with petroleum ether. For the identification of the fatty acid methyl esters, a Barber-Colman Series 5000 chromatograph with thermal conductivity detector was employed. The methyl esters derivatives were run on a 10-ft. x ¼ in. o.d. stainless steel column of 20% DEGS (diethylene glycol succinate) on 60–80 mesh Gas Pack S; the carrier gas was helium at 50 ml/min. The injector, column, and detector temperatures were 315C, 195C, and 305C respectively.

The lipid extract from 38.5 g of lyophilized cells amounted to 4.2 g or a recovery of 10.9%. The saponification number, glycerol percentage (anhydrous basis), iodine number (Wijs), and average molecular weight calculated on the basis of triglyceride were 205, 9.9, 76.8, and 275 respectively. The methyl esters of the fatty acids were submitted to gas chromatography, and the various peaks were identified on the basis of their retention times as compared with internal standards of high purity. The

TABLE I

Fatty Acid Composition of *Pullularia pullulans,* Based on Gas Chromatography of Methyl Esters (DEGS Column).

Tentative fatty acid identification	Retention time in minutes	Relative fatty acid distribution (area %)
n–Tetradecanoic	2.4	0.5
C_{15}–Monounsaturated	3.2	0.1
n–Pentadecanoic	3.5	Trace
n–Hexadecanoic	4.6	24.7
C_{16}–Monounsaturated	5.6	4.4
n–Heptadecanoic	6.5	0.4
C_{17}–Monounsaturated	7.5	0.1
n–Octodecanoic	9.2	6.3
C_{18}–Monounsaturated	10.2	47.8
C_{18}–Diunsaturated	12.6	14.8
C_{18}–Triunsaturated	18.6	0.9

absence of *trans*-unsaturation was confirmed by infrared spectra. The unsaturated fatty acids, which amounted to 68.1%, constituted mainly the monounsaturated C_{18} acid (47.8%; Table I). Hexadecanoic acid (24.7%) was the main saturated member; the ratio of unsaturated to saturated components was 2:1. On the basis of many exploratory runs, good reproducibility of the data was achieved by the use of definite concentrations of glucose as well as several nutrient salts as specified. It might be mentioned that qualitative tests were positive for α and β carotenes but negative for vitamin D_2.

Certain similarities in regard to composition and yields are apparent among the lipids of *P. pullulans* and those of other yeasts, notably *Saccharomyces cerevisiae*. The amount of lipid extracted from *P. pullulans* on a dry-weight basis was 10.5–11.0%, a range which is consistent with that found by Hanahan et al. (2) and Kahane et al. (3) for *S. cerevisiae*, and with the findings by Cullimore and Woodbine (4) for several strains of *Lipomyces starkeyi*.

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An in vitro Approach to the Mechanism of Cholesterol Deposition in vivo

ATHEROSCLEROSIS is a condition characterized by "a variable combination of changes of the intima of arteries consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue, and calcium deposits . . . . ." (1). Prominent among the accumulation of lipids are neutral fat and cholesterol. Although the development of atherosclerosis may be followed histologically, such studies offer little in the way of a mechanism for the pathology that is observed. A better understanding of the etiology of atherosclerosis might be achieved by the availability of in vitro models that would mimic at least some of the changes observed in the phenomenon of atherogenesis. The studies reported in this communication describe an approach to such a model since they demonstrate one experimental condition where localized concentrations of cholesterol, at least approaching crystallization, accumulate on the surface of a relatively inert material, Permutit, from triglyceride solution.

First, ^{14}C-cholesterol of relatively low activity (140 cpm/mg, 280 dpm/mg) was weighed out in various amounts (10 to 90 mg) into screw-capped glass test tubes. Permutit (Eimer and Amend, according to Folin) was added (200 or 500 mg) to the dry cholesterol. Various triglycerides (2 milliliters) were added to the tubes. The tubes were then stoppered and placed in an apparatus that rotated the tubes (60 rpm) at incubation temperature (37C). Following rotation with incubation (18-24 hr), the tubes were centrifuged (10 min) in a clinical centrifuge. Aliquots of the supernatant solutions (ca. 1 ml) were transferred by disposable pipettes to tared counting vials. After weighing, scintillation solution (10 ml, 4 g PPO [2,5-diphenyloxazole] and 30 mg POPOP (1,4-di-2[5-phenyloxazoyl]-benzene) per liter of toluene) was added to each vial, and the solutions were counted in a liquid scintillation spectrometer (Packard Tri-Carb). From the counts obtained and the radioactivity of the cholesterol used, the amount of cholesterol adsorbed at each equilibrium level of cholesterol in solution was readily calculated. The procedures which are summarized are essentially those described in previous papers concerned with various aspects of the solubility of cholesterol in triglycerides (2-4). The results obtained with a variety of triglycerides are illustrated by the data of Fig. 1. The amount of cholesterol adsorbed (cpm/ml) at two levels of Permutit (100 mg/ml and 250

absence of *trans*-unsaturation was confirmed by infrared spectra. The unsaturated fatty acids, which amounted to 68.1%, constituted mainly the monounsaturated C_{18} acid (47.8%; Table I). Hexadecanoic acid (24.7%) was the main saturated member; the ratio of unsaturated to saturated components was 2:1. On the basis of many exploratory runs, good reproducibility of the data was achieved by the use of definite concentrations of glucose as well as several nutrient salts as specified. It might be mentioned that qualitative tests were positive for α and β carotenes but negative for vitamin D_2.

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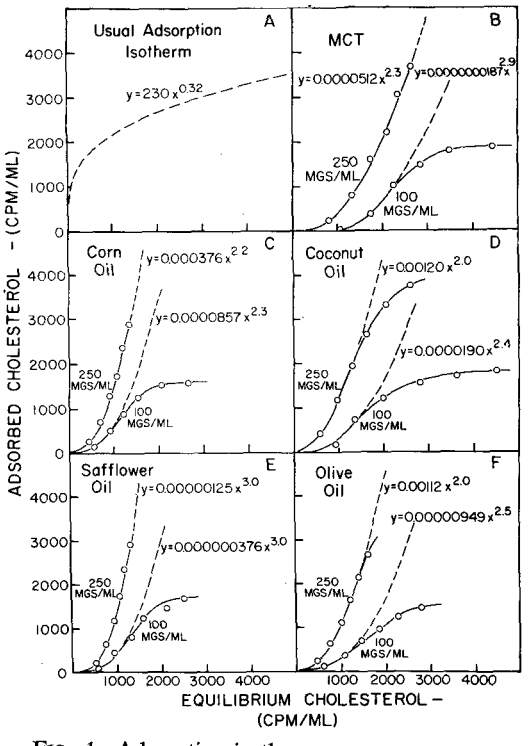

FIG. 1. Adsorption isotherms.

mg/ml) is plotted as a function of the equilibrium level of cholesterol (cpm/ml) in solution. Theoretical curves fitting the data have been calculated and are indicated in dotted lines.

In these experiments familiar Freundlich adsorption isotherms ($y = ax^n$, where $a > 1$ and $n < 1$), illustrated by an hypothetical curve (A, Fig. 1), were not obtained. Instead, curves that are "concave upwards" ($y = ax^n$, where $a < 1$ and $n > 1$) resulted. Such adsorption curves are typical of those encountered in "multiple layer" adsorption. In "multiple layer" adsorption one layer of adsorbate is first attracted with relatively low affinity to an adsorbing surface. The presence of this first adsorbed layer renders the surface more attractive to a second layer than to the first layer, and the presence of a second layer renders the surface more attractive to a third layer than to the second layer and so on, in sort of a "bootstrap" or vicious-circle manner. Obviously there are limits to the amount of cholesterol that can be adsorbed by a given amount of Permutit, and this level is easily reached by 100 mg/ml of Permutit as seen by the results of the figure.

The accumulation of layer on top of layer at a surface has some of the features of crystallization. Presumably, had it been possible to use enough adsorbing agent in these experiments, adsorption would have continued until the adsorbed layers crystallized.

Using the data for safflower oil and the higher level of Permutit (250 mg/ml), as an example, it was of interest to differentiate the theoretical curve fitting the data to yield an equation that describes the rate at which cholesterol is adsorbed by Permutit as a function of the level of cholesterol in solution. The equation $y = 0.00000125\ x^3$ on differentiation becomes $dy/dx = 0.00000375\ x^2$.

From this differentiated equation a number of interesting calculations can be made. For example, if an infinite capacity of 250 mg/ml of Permutit for adsorption is assumed, at a cholesterol level of only 1550 cpm/ml, which is a cholesterol concentration of only 1.12%, for every increment of cholesterol added to the solution 90% would be adsorbed on the Permutit. In the same way it can be shown that from an equilibrium mixture where the cholesterol concentration is approaching saturation, which is a cholesterol concentration of only 3.36%, for every increase in cholesterol concentration 99% of the increment would be adsorbed by the Permutit.

Although to postulate too strict an analogy between the adsorption of cholesterol on an inert adsorbing agent, such as Permutit, and the accumulation of cholesterol on tissue components may be unwarranted, it is not too far-fetched to suppose that there exist tissue components on which cholesterol may be readily adsorbed in multiple layers that eventually leads to crystallization. Such an hypothesis seems especially plausible in view of the facility with which cholesterol is adsorbed on Permutit. Separate experiments have shown that cholesterol is not adsorbed to any appreciable extent by Norit from triglyceride solution. Since the surface area per gram is much greater with Norit, as compared with Permutit, it is apparent that the nature of the adsorbing surface is of more significance in the adsorption of cholesterol from solution in triglycerides than is the actual surface area involved. The nature of the solvent from which cholesterol is adsorbed on Permutit is also of considerable significance with respect to the type of adsorption isotherm obtained. For example, cholesterol is well adsorbed on Permutit from solution in heptane, but the usual type of adsorption isotherm (A, Fig. 1) is obtained with a theoretical curve fitting the experimental points that is described by $y = 859\ x^{0.33}$ ($a>1$, $n<1$). A

curve of this shape is indicative of monolayer absorption that is most commonly seen in adsorption studies.

A second unexpected finding from these studies is that cholesterol is adsorbed better from solution in a "hypocholesterolemic" triglyceride, such as corn oil or safflower oil, than from a "hypercholesterolemic" triglyceride, such as coconut oil. The significance, if any, of this observation is not immediately apparent.

ACKNOWLEDGMENTS

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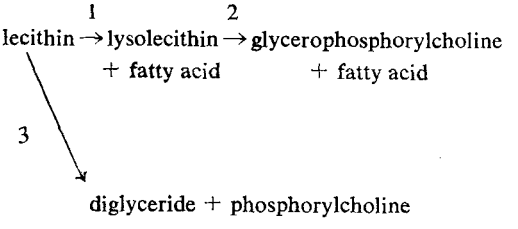
On the Phospholipases of Yeast

IN A PREVIOUS STUDY on the conversion of lecithin and lysolecithin by yeast extracts we obtained evidence for the participation of phospholipase A (EC 3.1.1.4) and lysophospholipase (EC 3.1.1.5) activities (1,2) (reactions 1 and 2, respectively). On the other hand Harrison and Trevelyan (3) attributed a breakdown of phospholipids occurring during a drying of yeast to the action of phospholipase C (EC 3.1.4.3) activity (4) (reaction 3).



In view of these differences, it seemed desirable to obtain more conclusive information about the phospholipases present in yeast.

Yeast *(Saccharomyces cerevisiae)* was cultured on sterilized malt extracts containing 1% glucose (w/v). After incubation for 20 hr at 30C under strong aeration the cells were harvested by centrifugation. Enzyme preparations (yeast extracts) were obtained by stirring yeast cells in three volumes (w/v) of 0.1 M Tris buffer pH 7.2 for 2 hr at room temperature. After centrifugation at 3000 × *g* a clear slightly yellow extract was obtained. ^{32}P-labeled lecithin and lysolecithin were ultrasonicated in buffer and incubated with 1.0 ml of the yeast extract at 37C under continuous shaking. Reactions were stopped by the addition of chloroform-methanol (1:2, v/v). Hydrolysis products were separated into polar and apolar compounds, and identification of the water-soluble reaction product was done by paper chromatography, using as reference substances glycerophosphorylcholine and phosphorylcholine prepared from ^{32}P-lecithin by alkaline hydrolysis and phospholipase C (from *B. cereus*) degradation, respectively. The degradation product formed after incubation of both lecithin or lysolecithin with the yeast extract revealed only one spot in three solvent systems. The R_F values of the degradation product, glycerophosphorylcholine and phosphorylcholine respectively were: in propanol-ammonia-water (6:3:1, v/v/v), 0.44, 0.44 and 0.26; in picric acid-*t* butanol-water (1:20:5, w/v/v), 0.41, 0.45 and 0.60; in phenol-water, 0.90, 0.93 and 0.75. These results indicate a degradation pathway according to reactions 1 and 2. Formation of phosphorylcholine could not be detected even by using whole yeast cells rather than the extract. Neither the suspension of whole cells nor that of ultrasonicated cells showed phospholipase C activity. Other modifications such as variation in the pH of the medium (pH 4.6 and 7.2), or addition of Ca-ions and ether did not allow phospholipase C activity to be detected.

However, the results reported by Harrison and Trevelyan and by Hoffmann-Ostenhof et al. (4) were obtained on yeast that had been dried previously. For that reason the experiments were repeated with extracts or cells of

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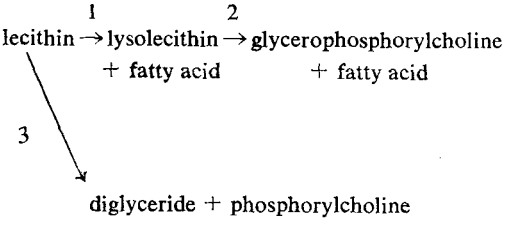

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yeast reconstituted after drying to constant weight in an air atmosphere at 30C. The only lipolytic activity detectable was that producing glycerophosphorylcholine and fatty acids. The formation of the latter was investigated by incubating 1.0 ml of an extract of dried yeast with 100 mμmole of synthetic 1-stearoyl-2-[1-^{14}C]-oleoyl-glycero-3-phosphorylcholine (spec. act. 0.38 μc/μmole). After incubation the lipids were extracted and subjected to thin-layer chromatography on silica using petroleum ether-diethylether-formic acid, (60:40:1.5, v/v/v) as the solvent system. Radioactivity as measured in eluates of the spots in a liquid scintillation spectrometer was found mainly in the fatty acid (86.5%) and fatty acid ester (12.2%) fractions whereas diglycerides accounted for only 1.3% of the total radioactivity in the hydrolysis products.

In order to obtain some further information about the processes that occur during drying of yeast, the cells were cultivated in a medium containing 1% glucose and [1-^{14}C]-acetate (60 μc/100 ml, spec. act. 188 μc/mg). Cells were harvested by centrifugation and washed twice with a 0.1 M Na-acetate solution in saline. A part of the culture was extracted directly, whereas another part was first dried at 30C until a constant weight was reached (about 36 hr). In this procedure 2 g of wet yeast (dried between filter paper) gave 0.62 g dried yeast, and after extraction 44 mg lipids (7.1% of dry weight) were obtained. The distribution of the radioactivity between the yeast lipids gave the following pattern, before and after drying respectively: phospholipids, 58.1 and 28.1%; monoglycerides, 1.8 and 1.5%; diglycerides, 15.7 and 13.2%; fatty acids, 16.7 and 39.8%; triglycerides, 4.4 and 5.4%; fatty acid esters, 2.0 and 8.6%; sterol esters, 1.8 and 3.4%. The large decrease in phospholipid radioactivity appears to be balanced almost entirely by an increase in the radioactivity of the fatty acids and the fatty acid esters, whereas diglycerides remain fairly constant. The fatty acid esters are most likely formed during the extraction of the lipids with chloroform-methanol mixtures.

The results available so far point to the presence of a phospholipase A and a lysophospholipase in yeast, as shown by identification of the water-soluble as well as the lipid-soluble degradation products. These results were completely confirmed by the observation that the decrease in yeast phospholipid content under our conditions of drying of yeast is balanced by an increase in fatty acids rather than by an increase of diglycerides.

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[Received Jan. 9, 1967]

Determination of Unsaturation in Oils in the Presence of Saturated Aldehydes

DURING STUDIES on the preparation of aldehyde oils (4) it was desirable to determine unsaturation in the presence of aldehyde groups. The Wijs iodine value method gave results which showed more than the true amount of unsaturation, as indicated by GLC data. The quantitative determination of unsaturation in oils by the addition of halogens is reported to be considerably affected by the presence of some carbonyl compounds (1). Therefore the hydrogenation method of determining unsaturation developed by Brown et al. (2) and improved by Miwa et al. (3) was investigated as a means of determining unsaturation in aldehyde oils. This method employs a platinum catalyst with in situ generation of hydrogen from sodium borohydride solution.

Hydrogenation of unsaturated vegetable oils by the Brown method is rapid, and a sharp end-point for the reaction is obtained after about 10 minutes. However aldehydes were found to undergo a much slower reduction by this method (Figure 1), and the reaction continues well beyond the time required for saturation of double bonds.

A mixture of an unsaturated vegetable oil and an aldehyde showed a rapid initial rate of reduction, and after a fairly sharp break a much slower reaction as aldehyde reduction continued. A plot was made of sodium boro-

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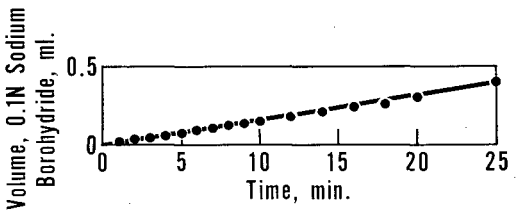

FIG. 1. Titration curve for a sample of pure pelargonaldehyde.

hydride reagent used versus time, and the linear part of the curve (aldehyde reduction) was extrapolated to zero time as shown in Figure 2. The intercept was taken as the quantity of hydrogen (borohydride reagent) which reacted only with the unsaturation. The hydrogen iodine value (HIV) then could be calculated from the intercept value.

Unsaturated aldehyde oils analyzed by the titration curve method gave HIV's in agreement with values indicated by GLC data. For a more reliable measure of the accuracy of the graphical method, samples were prepared for analysis by combining weighed amounts of saturated aldehydes and unsaturated triglycerides or methyl esters of known HIV. These were then hydrogenated, and the volume of borohydride reagent was read at one-minute intervals and plotted to give a curve. After extrapolation of the secondary part of the curve to zero time, HIV's were calculated. Those shown in Table I were obtained on samples taken from prepared mixtures of pelargonaldehyde and methyl oleate; values in Table II, from mixtures of soybean oil and methyl azelaaldehydate. In these tests the titration curve method gave a good degree of accuracy and repeatability. It was not necessary to wait for completion of the reduction reaction of one aldehyde sample before adding a second sample to the same solution for an HIV determination. The slope of the curve

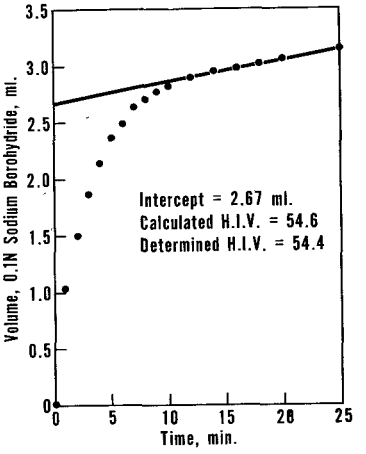

FIG. 2. Hydrogen iodine value (HIV) obtained from a titration curve for a mixed sample of pelargonaldehyde and methyl oleate.

for the secondary reaction for the second sample was somewhat more steep than for the first sample, but this did not interfere with the determination.

The aldehydes used in these studies did not adversely affect catalyst activity. After reduction of a sample containing an aldehyde, the catalyst showed no lowering of activity for reduction of an unsaturated oil containing no aldehydes.

Crotonaldehyde (Eastman No. 1878) consistently yielded HIV's which were 20% higher than theory. Since this is a conjugated enal, it is believed the initial rate of aldehyde reduction may have been accelerated, but after saturation of the C-C bond it apparently proceeded normally. A high initial aldehyde reduction rate would lead to erroneously high values for unsaturation.

An ideal catalyst would have an even higher selectivity than the platinum catalyst used, *i.e.*, effective on unsaturation without any aldehyde reduction. Palladium gave a slower rate for

TABLE I

Hydrogen-Iodine Values Determined for Mixtures Prepared from Methyl Oleate and C_9 Aldehyde

Methyl oleate, %	Determined hydrogen-iodine value				Theoretical hydrogen-iodine value
				Average	
14.2	11.7,	11.4,	11.8	11.6	12.1
32.5	30.3,	29.8,	29.9	30.0	27.7
60.5	55.0,	55.2,	54.4	54.9	54.6
85.1	75.7,	74.7,	75.8	75.4	72.6

TABLE II

Hydrogen-Iodine Values Determined for Mixtures Prepared from Soybean Oil and Methyl Azelaaldehydate

Soybean oil, %	Determined hydrogen-iodine value				Theoretical hydrogen-iodine value
				Average	
24.9	32.2,	33.0,	35.3	33.5	33.8
49.6	66.0,	64.7,	66.2	65.8	65.2
75.0	98.0,	95.9,	98.2	97.4	98.7

both reactions and was therefore less suitable than platinum. Other possible catalysts were not investigated.

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[Received March 1, 1967]

Disc Electrophoresis of Human and Animal Serum Lipoproteins

DISC ELECTROPHORETIC PATTERNS which were obtained with rat serum were strikingly different from human serum patterns, indicating the presence of several previously unobserved lipoprotein components (1,2). To date, the serum lipoprotein patterns of other species, using polyacrylamide gel, have not been reported. Since chickens, rabbits, and mice are commonly used in studies on lipid metabolism, it was considered worthwhile to investigate the type and number of electrophoretically resolvable lipoprotein components in the sera of these species and to compare them with those of rat and human serum.

The animals, rats (Holtzman), chickens (Columbian-New Hampshire cross), rabbits (New Zealand), and mice (CF#1) were maintained on normal, commercially available diets (Purina Labena, Poultry ration, Purina rabbit chow checkers, and Purina Labena respectively). The prestaining procedure and the electrophoretic method were as described earlier (1) except that whole serum was substituted for whole plasma because gelation was sometimes encountered with prestained plasma. In these experiments, individual nonfasting animal sera were used except in the case of the mouse, where pooled sera from nonfasting mice was used. However, in all cases, at least three separate samples were used, and the patterns presented (Fig. 1) are typical of those obtained for each species. Densitometer tracings of the resolved gel patterns were obtained by using a Model E Microdensitometer (Canalco, Bethesda, Md). The densitometer traces. rather than photographs, have been presented because they provide a measure of quantitation of the separated bands.

The present experiments have indicated the presence of 2–6 major and 2–6 minor lipoprotein components in the five species. The major components consist of at least one slow component close to the main gel-spacer gel interface and at least one fast component which was located just behind the albumin component. On the basis of the electrophoretic patterns obtained with ultracentrifugally isolated human and rat serum lipoproteins, these slow and fast components have been identified (1,2) as low-density and high-density lipoproteins respectively (LDL and HDL). At gel concentrations of 3.75% and above, the serum albumin is generally resolved from the high-molecular-weight, high-density lipoprotein. The albumin is detected as a brown or grey component because it binds a polar, brown impurity present in Sudan Black B (2). In some cases (for example, when the albumin carries certain lipids, such as free fatty acids, and when excessive amount of the impurity is not present in the dye) the albumin component is detected as a light green or blue band.

With chicken serum, one LDL component and one HDL component were prominently seen at all gel concentrations (Fig. 1). The HDL was apparently the principal lipoprotein species in this serum since the ratio of the peak areas of HDL to LDL was observed to be 3.4. With 7.5% gel, 4–6 minor components of intermediate mobilities between the LDL and HDL components were also observed. Two of these minor components were only partially resolved from the main HDL component and may therefore be part of the HDL fraction. In mouse serum, the patterns indicated the presence of 2–4 LDL components and 2 HDL components (Fig. 1). The total intensity of HDL components was greater than the LDL components. However, in 3 75% gel, the ratio of the peak areas of HDL to LDL was lower than that in chicken serum. With rabbit serum, the principal components were one LDL component and one HDL component and 2–3 minor components of intermediate mobilities (Fig. 1). The albumin component was detected as a grey band at all gel concentrations. In 3.75% gel, the ratio of the peak areas of HDL to LDL was also lower than that ob-

both reactions and was therefore less suitable than platinum. Other possible catalysts were not investigated.

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Disc Electrophoresis of Human and Animal Serum Lipoproteins

DISC ELECTROPHORETIC PATTERNS which were obtained with rat serum were strikingly different from human serum patterns, indicating the presence of several previously unobserved lipoprotein components (1,2). To date, the serum lipoprotein patterns of other species, using polyacrylamide gel, have not been reported. Since chickens, rabbits, and mice are commonly used in studies on lipid metabolism, it was considered worthwhile to investigate the type and number of electrophoretically resolvable lipoprotein components in the sera of these species and to compare them with those of rat and human serum.

The animals, rats (Holtzman), chickens (Columbian-New Hampshire cross), rabbits (New Zealand), and mice (CF#1) were maintained on normal, commercially available diets (Purina Labena, Poultry ration, Purina rabbit chow checkers, and Purina Labena respectively). The prestaining procedure and the electrophoretic method were as described earlier (1) except that whole serum was substituted for whole plasma because gelation was sometimes encountered with prestained plasma. In these experiments, individual nonfasting animal sera were used except in the case of the mouse, where pooled sera from nonfasting mice was used. However, in all cases, at least three separate samples were used, and the patterns presented (Fig. 1) are typical of those obtained for each species. Densitometer tracings of the resolved gel patterns were obtained by using a Model E Microdensitometer (Canalco, Bethesda, Md). The densitometer traces. rather than photographs, have been presented because they provide a measure of quantitation of the separated bands.

The present experiments have indicated the presence of 2–6 major and 2–6 minor lipoprotein components in the five species. The major components consist of at least one slow component close to the main gel-spacer gel interface and at least one fast component which was located just behind the albumin component. On the basis of the electrophoretic patterns obtained with ultracentrifugally isolated human and rat serum lipoproteins, these slow and fast components have been identified (1,2) as low-density and high-density lipoproteins respectively (LDL and HDL). At gel concentrations of 3.75% and above, the serum albumin is generally resolved from the high-molecular-weight, high-density lipoprotein. The albumin is detected as a brown or grey component because it binds a polar, brown impurity present in Sudan Black B (2). In some cases (for example, when the albumin carries certain lipids, such as free fatty acids, and when excessive amount of the impurity is not present in the dye) the albumin component is detected as a light green or blue band.

With chicken serum, one LDL component and one HDL component were prominently seen at all gel concentrations (Fig. 1). The HDL was apparently the principal lipoprotein species in this serum since the ratio of the peak areas of HDL to LDL was observed to be 3.4. With 7.5% gel, 4–6 minor components of intermediate mobilities between the LDL and HDL components were also observed. Two of these minor components were only partially resolved from the main HDL component and may therefore be part of the HDL fraction. In mouse serum, the patterns indicated the presence of 2–4 LDL components and 2 HDL components (Fig. 1). The total intensity of HDL components was greater than the LDL components. However, in 3 75% gel, the ratio of the peak areas of HDL to LDL was lower than that in chicken serum. With rabbit serum, the principal components were one LDL component and one HDL component and 2–3 minor components of intermediate mobilities (Fig. 1). The albumin component was detected as a grey band at all gel concentrations. In 3.75% gel, the ratio of the peak areas of HDL to LDL was also lower than that ob-

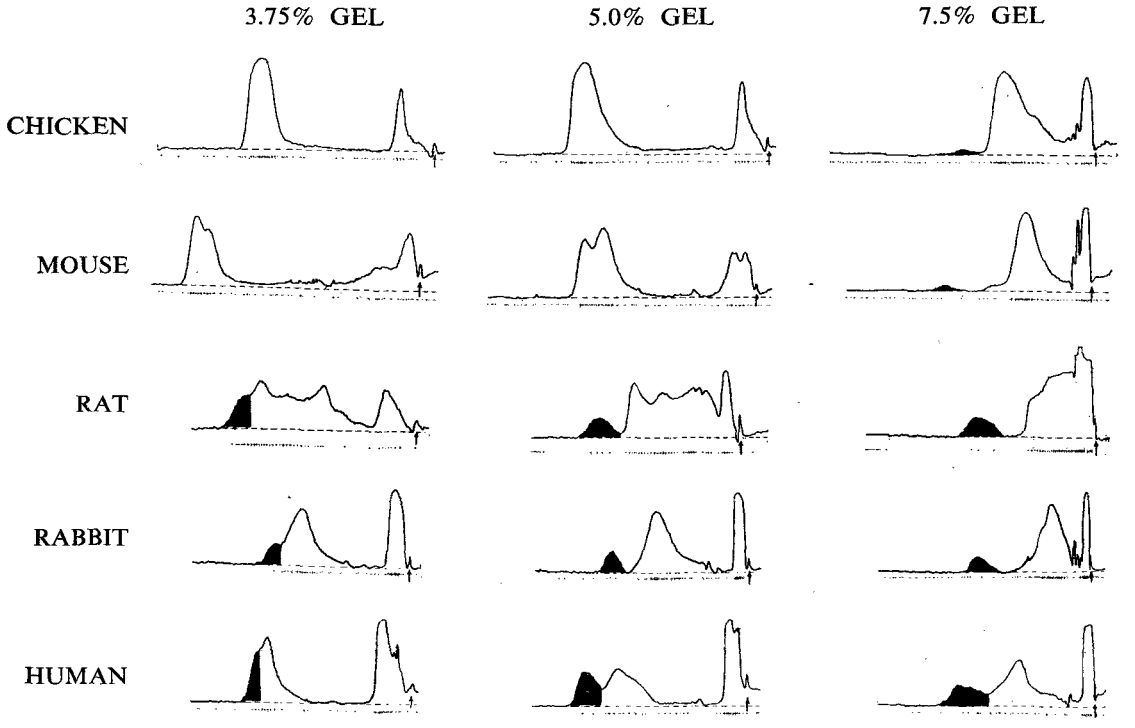

FIG. 1. Typical disc electrophoretic patterns of chicken, mouse, rat, rabbit, and human serum lipoproteins at three different gel concentrations, as indicated. Migration from right to left with the arrows indicating the origin (main gel-spacer gel interface). The albumin component, where detected by Sudan Black B stain, indicated as a solid area.

served in the case of chicken serum, consistent with literature values (3).

For purposes of comparison, patterns of a normal human male serum at three main gel concentrations are presented (Fig. 1). One to three closely spaced LDL components and 1–3 HDL components were observed. The albumin component separated as a light green and brown band in 5% and 7.5% gel respectively. The HDL resolved into 2–3 components in the 5% gel and possibly into 3–4 components in the 7.5% gel. In 3.75% gel, the 0.9 ratio of the peak areas (uncorrected for albumin) of HDL to LDL was considerably lower than for the other species. In the serum of rats fed Purina labena, between 4–6 lipoprotein components were visible at all gel concentrations (Fig. 1) and confirmed earlier results, where a semi-synthetic diet was employed (1).

At 5 and 7.5% gel concentrations, the low-density lipoproteins remain as tightly packed bands close to the spacer gel-main gel interface and apparently resulted in the variations observed in the LDL peak areas at different gel concentrations. For example, even though identical quantities of the same prestained sample was used, the human LDL peak area decreased from 408 mm^2 using 3.75% gel, to 360 mm^2 using 5% gel, and to 248 mm^2 using 7.5% gel. The ratios of the peak areas (uncorrected for albumin) of HDL to LDL at these three gel concentrations were 0.9, 1.3, and 1.9 respectively, indicating that peak areas of the various components which were separated at different gel concentrations must be interpreted with caution. Furthermore, satisfactory correlation between lipoprotein concentration and stain intensity has not been observed because the dye uptake is apparently dependent upon the lipid composition of the lipoproteins (1).

The serum lipoproteins of several species have been previously resolved into 2–4 components by using paper electrophoresis, moving boundary electrophoresis, or ultracentrifugation (3,4). The potentially increased resolution observed may be ascribed to differences in the pore sizes of the gels which enabled closely related lipoproteins to be separated. Although

high-density lipoproteins were apparently the predominant species in the sera of chicken, rabbit, and mouse, the serum lipoprotein patterns of these species were similar to the human rather than to the rat. In all species except rat, when 3.75% gel was used, there was an absence of major lipoprotein components of mobilities intermediate to those of LDL and HDL. Other data indicate that a significant portion of the intermediate components observed with rat serum is probably attributable to the HDL_1 ($d = 1.050$) fraction (2). Therefore the HDL_1 fraction may be absent or present at low concentration in the sera of other species. Alternatively the characteristics of the serum HDL_1 fraction may vary among species.

ACKNOWLEDGMENTS

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[Received Nov. 15, 1966]

Effect of Insulin on the Oxidative Desaturation of α-Linolenic, Oleic and Palmitic Acids

GELLHORN AND BENJAMIN (1) showed that the oxidative desaturation of stearic to oleic acid is depressed in the microsomes of alloxan-diabetic rats. The same defect was also found by Mercuri, Peluffo and Brenner (2) in the conversion of linoleic into γ-linolenic acid in the liver of the same type of animals. When the diabetic rats were injected with insulin the desaturating activity for both acids was recovered. Insulin, however, had no effect in vitro. In this study, evidence has been obtained that the oxidative desaturation of α-linolenic to octadeca-6,9,12,15-tetraenoic acid, oleic to octadeca-6,9-dienoic acid and palmitic to palmitoleic acid are also depressed in the liver microsomes of alloxan-diabetic rats.

Alloxan-diabetes was induced in two-month-old female rats from the strain of the Institute. Alloxan monohydrate (50 mg/kg, Nutritional Biochemical Corp., Cleveland, Ohio) was injected intravenously. Animals were considered diabetic when fasting blood-glucose levels exceeded 300 mg/100 ml. The rats were assembled in groups of five animals each. A first group was diabetic. A second group was formed by diabetic rats injected twice a day for the 48 hr prior to sacrifice with 15 units of glucagon-free crystalline insulin (Eli Lilly, Buenos Aires) and 10 ml of 5% glucose in isotonic saline. Two hours before being sacrificed, these animals were again injected with 15 units of insulin. A third group did not receive any treatment at all. A week prior to sacrifice all the animals received a semisynthetic fat-deficient diet (3) supplemented with 50% fat-free casein in order to decrease the intracellular content of essential fatty acids.

The activity of the oxidative desaturating enzymes of the liver microsomes was individually measured for each rat using 1-^{14}C α-linolenic acid (22.0 mc/mM, 89% radiochemically pure, Radiochemical Center, Amersham, England), 1-^{14}C oleic acid (32.5 mc/mM, 99% radiochemical purity, Radiochemical Center, Amersham, England) and 1-^{14}C palmitic acid (29.6 mc/mM Calbiochem, Los Angeles, Cal.) as substrates. The microsomes were separated by differential centrifugation in a Spinco L2 ultracentrifuge at 140,000 × *g* (4). Five milligrams of microsomal protein and 0.2 ml supernatant were incubated for 20 min in O_2 at 35 C with 10 mμmoles of the labeled acids in 0.6 ml of a 0.15 M KCl – 0.25 M sucrose solution, containing in μmoles: ATP 0.8, CoA 0.04, NADPH 0.16, NADH 0.33, $MgCl_2$ 3, glutathione 0.9, NaCN 0.6, NaF 25, nicotinamide 0.2 and phosphate buffer (pH 7) 25. The acids added were dissolved in 10 μl propylene glycol. The reaction was stopped by

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TABLE I

Effect of Insulin on the Oxidative Desaturation of 1-^{14}C α-linolenic, 1-^{14}C oleic and 1-^{14}C palmitic.

Incubated acid	Diabetic	Normal	Treated diabetic
Linolenic	22.3 ± 0.2	31.6 ± 1.5 P < 0.01	28.2 ± 1.9 P < 0.05
Oleic	3.1 ± 0.2	5.1 ± 0.4 P < 0.001	5.7 ± 0.2 P < 0.001
Palmitic	22.7 ± 1.6	33.2 + 2.1 P < 0.02	41.5 ± 1.7 P < 0.001

Experimental condition and incubation as described in the text. Results expressed as percentage of labeled acid desaturated. Values given are means of 5 animals analyzed in duplicate ± standard error of the means. Probability values are related to the diabetic rat. Mean blood glucose level in mg/100 ml at the time of sacrifice were: diabetic, 360+18; normal 103+0; treated diabetic, 106+16.

addition of 10% alcoholic KOH and the fatty acids recovered by saponification. The fatty acids were esterified with methanolic 3 N HCl for 3 hr at 60C and the composition and radioactivity distribution determined by gas-liquid-radiochromatography in a Pye apparatus with ionization detector and proportional counter. The samples were analyzed in columns packed with 12% polyethylene glycol succinate on Chromosorb W (80-100 mesh) at 185C. The structures of the labeled acids synthesized were proved by reductive ozonolysis (4). When α-linolenic acid was incubated, the radioactivity was only found in α-linolenic and octadeca-6,9,12,15-tetraenoic acid. When oleic acid was assayed the radioactivity appeared only in oleic and octadeca-6,9-dienoic acid and when palmitic acid was tested, labeling was shown in palmitic and palmitoleic acid and only traces in hexadecadienoic acid.

The different oxidative desaturating activity of the microsomes of the three groups of rats expressed as the percent conversion for each of the three acids tested (Table I) shows that in alloxan diabetes microsomal enzymatic activity is depressed for several conversions. These include stearic to oleic acid, linolenic to γ-linolenic acid, α-linolenic to octadeca-6,9,12, 15-tetraenoic acid, oleic to octadeca-6,9-dienoic acid and palmitic to palmitoleic acid. All of these defects were corrected by insulin treatment in only two days. However, the recovery of the activity was not the same for the different desaturating reactions. The desaturates for palmitic and stearic acids gave higher responses to insulin than the enzyme for desaturation of linoleic or α-linolenic acid.

It follows from experimental evidence presented that polyunsaturated fatty acids such as eicosatrienoic, arachidonic, eicosapentaenoic, docosahexaenoic acid, etc., would be decreased in the diabetic animal. This defect would be only partially corrected by feeding seed oils. These oils provide only oleic, linolenic and α-linolenic acid that would not be converted in the normal manner to higher polyunsaturated acids by the diabetic animal. The mentioned polyunsaturated acids play an important role in building "normal" phospholipids, in the permeability of membranes, in the production of energy, in the electron transport chain, in the synthesis of prostaglandin, in reproduction, etc. In consequence, many of the disturbances associated with diabetes (for example, vascular lesions) may be a consequence of the depressed desaturating activity of the microsomes.

TABLE II

Fatty Acid Composition of the Liver Lipids of Alloxan-Diabetic Rats (Percent of the Total)

Fatty acid[a]	Control	Diabetic
16:0	23.9	21.7 - 24.6
18:0	19.9	22.0 - 24.2
18:1	15.2	14.0 - 16.2
18:2	16.4	20.1 - 23.0
20:4	21.9	12.8 - 15.8

[a] Other components not tabulated account for 100%.

A decrease in arachidonic acid and an increase in linoleic acid content was actually shown in the fatty acid composition (Table II) of the total liver lipids of alloxan-diabetic rats after only 2 weeks of alloxan injection.

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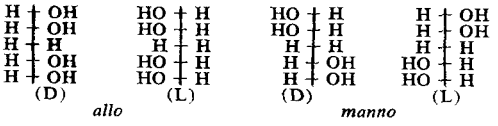
LETTERS TO THE EDITOR

Naming Diastereoisomeric Polyhydroxystearates

Sir: The diastereoisomeric 9,10-dihydroxystearates are best designated as *threo* and *erythro* isomers, depending on their relationship to the aldotetrose sugars threose and erythrose. Hydroxylation of linoleic acid or of linelaidic acid yields two *erythro*-9,10-*erythro*-12,13-tetrahydroxystearic acids or two *threo*-9,10-*threo*-12,13 isomers, but until recently it was not possible to assign a more detailed configuration to these four acids or to the eight optically active isomers obtained from vernolic acid (Bharucha and Gunstone, J. Chem. Soc. 1611, 1956). Recent contributions to this journal (Wood et al., Lipids *1*, 399, 1966; Morris and Wharry, Lipids *1*, 41, 1966) have now settled this for the 9,10,12,13-tetrahydroxystearic acids and for the 9,10,12-trihydroxystearic acids derived from ricinoleic and ricinelaidic acid.

The repeated use of the terms *threo* and *erythro* for these polyhydroxy acids is clumsy, and we propose that the isomeric trihydroxy acids be related to the aldopentoses and the isomeric tetrahydroxy acids to the aldohexoses. On this basis the two *erythro, erythro* tetrahydroxystearic acids are *allo* and *manno* isomers.



In a Fischer projection the more oxygenated end of the structure is placed uppermost and therefore, in these polyhydroxy monobasic acids, C(9) will appear above C(12) and C(13). The direction of the lowest hydroxyl group fixes the configuration as D or L, as shown above. Correlation of available data allows the assignments in the following tables to be made for the 9, 10, 12-trihydroxystearic acids and the 9, 10, 12, 13-tetrahydroxystearic acids. Configurations cannot be assigned to the optically active 9, 12, 13-trihydroxystearic acids (Gunstone and Morris, J. Sci. Fd. Agric. *10*, 522, 1959) without further experimental work since it is not yet possible to relate configuration with melting point only, when two CH_2 groups separate a pair of the hydroxyl groups.

TABLE I
9, 10, 12-trihydroxystearic acids (mp)

D—*arabino*	(9R, 10R, 12R)	85°
D—*xylo*	(9S, 10S, 12R)	106
D—*ribo*	(9S, 10R, 12R)[a]	111
D—*lyxo*	(9R, 10S, 12R)[a]	136

[a] Incorrectly designated by Wood et al.

TABLE II
9, 10, 12, 13-tetrahydroxystearic acids (mp)

	rac	L	
ido	148°	148	(9S, 10S, 12S, 13S)
galacto	126	122	(9R, 10R, 12S, 13S)
gulo	164	165	(9R, 10S, 12S, 13S)
talo	126	112	(9S, 10R, 12S, 13S)
	rac	D	
gluco	164	156	(9S, 10S, 12S, 13R)
altro	142	130	(9R, 10R, 12S, 13R)
manno	174	177	(9R, 10R, 12S, 13R)
allo	164	156	(9S, 10R, 12S, 13R)

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Erratum

"Phosphonolipids. IX. Phosphonolipid Metabolites: Synthesis of L-α-Glyceryl-(2-trimethylammoniumethyl)phosphonate," by Erich Baer and Ranga Robinson. *Lipids*, March, 1967. On page 195, column 1, line 15 should begin, "$[\alpha]_D$–0.6° in water. . . ." On the same page, column 2, line 6 should read, "$[\alpha]_D^{24}$ –1.5° in water. . . ." The name of the co-author, DR. RANGA ROBINSON, was inadvertently omitted from the signature of this communication.

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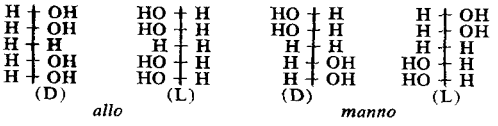

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Influence of Medium-Chain Triglycerides on Lipid Metabolism in the Rat

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ABSTRACT

Lipid metabolism was studied in rats fed diets containing corn oil, coconut oil, or medium-chain triglyceride (MCT), a glyceride mixture containing fatty acids of 8 and 10 carbons in length. The ingestion of MCT-supplemented, cholesterol-free diets depressed plasma and liver total lipids and cholesterol as compared with corn oil-supplemented diets. In rats fed cholesterol-containing diets, plasma cholesterol levels were not influenced by dietary MCT, but liver cholesterol levels were significantly lower than in animals fed corn oil. In vitro cholesterol synthesis from acetate-1-^{14}C was lower in liver slices of rats that consumed MCT than in similar preparations from corn oil-fed rats. Studies of fatty acid carboxyl labeling from acetate-1-^{14}C and the conversion of palmitate-1-^{14}C to C_{18} acids by liver slices showed that chain-lengthening activity is greater in the liver tissue of rats fed MCT than in the liver of animals fed corn oil. The hepatic fatty acid desaturation mechanisms, evaluated by measuring the conversion of stearate-2-^{14}C to oleate, was also enhanced by feeding MCT.

Adipose tissue of rats fed MCT converts acetate-1-^{14}C to fatty acids at a much faster rate than does tissue from animals fed corn oil. Evidence is presented to show that the enhanced incorporation of acetate into fatty acids by the adipose tissue of rats fed MCT represents de novo synthesis of fatty acids and not chain-lengthening activity. Data are also presented on the fatty acid composition of plasma, liver, and adipose tissue lipids of rats fed the different fats under study.

INTRODUCTION

THE HYPOCHOLESTEROLEMIC EFFECT of dietary medium-chain triglyceride (MCT), a triglyceride mixture containing approximately 75% caprylic (C_8) and 25% capric (C_{10}) acids, has received considerable attention in recent years. MCT, when substituted in the diet for glycerides containing fatty acids of longer-chain length, has been shown to depress serum cholesterol levels in the rat (1-4), dog (5), rabbit (6), and in man (7). The mechanism involved in the cholesterol depression induced by MCT remains to be elucidated.

Recently Kritchevsky and Tepper (4) have investigated the effect of dietary MCT on hepatic lipogenesis and cholesterogenesis in the rat. The incorporation of acetate-1-^{14}C into fatty acids, both in vivo and in vitro, was enhanced by MCT ingestion. The influence of MCT on acetate-1-^{14}C incorporation into cholesterol was less conclusive. MCT-fed rats incorporated less acetate into liver cholesterol in vivo than did animals ingesting a corn oil-supplemented diet; however liver slices from MCT-fed rats converted significantly more acetate-1-^{14}C into cholesterol than did slices from corn oil-fed animals. Consequently the influence of MCT on hepatic cholesterogenesis is not clear.

Kritchevsky and Tepper (4) and Reiser et al. (9) have suggested that the increased incorporation of acetate-^{14}C into fatty acids, induced by MCT ingestion, may reflect increased chain-lengthening activity rather than de novo synthesis of fatty acids. Suggestive evidence in support of this contention has been presented (9,10); however this point remains to be established.

The studies to be reported were undertaken in an effort to define further the metabolic alterations induced in various tissues as a consequence of the ingestion of MCT. These investigations were concerned with the effects of dietary MCT on fatty acid biosynthesis, elongation, and desaturation in liver and adipose tissue and on cholesterol synthesis in liver. The results obtained demonstrate that liver and adipose tissue respond to the ingestion of MCT differently, and the data further suggest a mechanism for the hypocholesterolemic effect of dietary MCT.

The principles of laboratory animal care, as

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TABLE I
Percentage Composition of Fatty Acids of Various Fats, Diet, and the Liver of a Control Animal

Fatty acid[a]	Corn oil	Coconut oil	MCT	Lab chow	Liver lipids of lab chow-fed rat
8:0	—	6.9	55.9	—	—
10:0	—	6.5	36.3	0.8	—
12:0	—	38.6	3.8	0.4	1.0
12:7?[b]	0.6	—	—	—	—
14:0	0.3	20.8	1.3	2.5	1.4
14:1	—	—	0.4	1.1	0.8
16:0	14.4	13.6	0.8	22.5	20.8
16:1	—	—	—	—	—
18:0	2.7	3.5	0.4	9.2	20.4
18:1	19.6	6.4	0.5	27.7	14.9
18:2	56.3	2.7	0.6	27.7	19.6
18:3	6.2	0.9	—	6.1	3.2
20:4	—	—	—	1.9	17.9

[a] The number preceding the colon represents the carbon chain-length, and the number following the colon indicates the number of double bonds.

[b] Unidentified fatty acid with a retention time of 12.7 carbons.

promulgated by the National Society for Medical Research, were observed.

EXPERIMENTAL PROCEDURE

Male Holtzman rats, weighing between 150 and 200 g and prefed Purina rat chow, were used in Experiments I-IV, and female Holtzman rats, weighing 216 g, were used for Experiment V. The animals were housed in stainless steel cages with raised wire floors and in a temperature- and humidity-controlled room (70F and 50% relative humidity). Food and water were supplied ad libitum, and food consumption and body weight were determined at weekly intervals. The basal diet used in these studies had the following percentage composition: vitamin-free casein, 20; L-cystine, 0.3; vitamin mix (Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, O.), 2.2; nonnutritive fiber, 4; salt mix (USP XIV), 4; corn oil, 2; glucose, 55.5; and fat supplement, 12. All diets therefore contained 14% lipid; the corn oil-supplemented diets contained 14% corn oil whereas the coconut oil and MCT (supplied by G. S. Knight, Drew Chemical Company, Boonton, N. J.) diets were composed of 12% of the fat under study plus 2% corn oil. The 2% corn oil was added to all diets to supply essential fatty acids since an essential fatty acid deficiency has been shown markedly to influence fatty acid synthesis (11). For comparison, values for the fatty acid composition of the fats used and for Purina rat chow are shown in Table I. The cholesterol-supplemented diets employed in these studies contained 2% cholesterol and 0.5% cholic acid added to the diets at the expense of glucose. The experimental diets were fed to groups of 7-10 rats for two or three weeks. The number of animals used and the duration of each experiment are indicated in the tables. In all experiments presented, animals fed corn oil served as controls for comparison with rats fed other lipid supplements.

At the end of each experiment the animals were sacrificed by decapitation, and blood was collected by funnel. The tissues to be used for metabolic studies and for chemical analysis were quickly removed. Liver slices were prepared with a Stadie-Riggs hand microtome from the left lateral lobe. Epididymal adipose tissue was used, and metabolic studies were carried out with segments of the thin peripheral portion, weighing approximately 100 mg. All incubations were carried out in calcium-free Krebs-Ringer bicarbonate buffer (12), pH 7.4, containing the appropriate substrates. For the study of fatty acid synthesis from acetate-1-^{14}C the buffer contained per ml 10 μmoles sodium acetate, 0.167 μC of acetate-1-^{14}C, and 5 μmoles glucose. Insulin (a gift from W. Bromer, Eli Lilly and Company, Indianapolis, Ind.), 0.1 unit per ml, was also added to flasks in which adipose tissue was incubated. For the study of palmitate-1-^{14}C and stearate-2-^{14}C utilization, calcium-free Krebs-Ringer phosphate buffer was fortified with 3% bovine serum albumin and 160 μmoles of fatty acid, 5 μmoles of glucose, and 0.25 μC of radioactive fatty acid per ml. The details of the incubation procedure, isolation of radioactive products, and counting procedures have been previously described (13).

The samples to be used for fatty acid analysis were extracted with chloroform: methanol (2:1), and the lipids were saponified by refluxing in 0.5 N methanolic KOH for 30 min. The nonsaponifiable fraction was extracted with

petroleum ether (bp 30-60C); the aqueous phase was acidified with N HCl, and the fatty acids were removed by repeated extraction with petroleum ether. The combined ether fraction was washed with water and dried over anhydrous Na_2SO_4. The solvent was carefully evaporated,[2] the acids were dissolved in 0.5 ml of 10% methanol in ether and methylated with diazomethane (14). The solvent was again carefully evaporated, and the fatty acid methyl esters were stored at –20C in petroleum ether until analyzed. Prior to analysis by gas-liquid chromatography, the methyl esters were dissolved in a minimum of carbon disulfide. Analyses were carried out on F & M models 609 and 810 research chromatographs equipped with hydrogen flame detectors. An 8-ft x ⅛ in. I.D. stainless steel column, packed with diethylene glycol succinate on Gaschrom P (100-200 mesh) (15), was used. The flow rate and temperature were adjusted to give a retention time of 10.3 min for methyl stearate. Equivalent retention-time values derived from standards were used to identify individual fatty acids. The results are expressed as area percentage; the peak areas were determined by triangulation (height x width at 0.5 height). In those studies in which individual fatty acids were collected for radioactivity determination, the F & M model 810 equipped with a stream splitter was used. Then 10% of the effluent was allowed to flow directly through the hydrogen flame detector; the remaining 90% of the effluent was directed to the heated collection port, and the fatty acids were collected in cooled glass capillaries. The isolated fractions were eluted with petroleum ether, and the radioactivity was determined by liquid scintillation, using a toluene scintillant solution (13). This isolation system was evaluated with palmitate-1-^{14}C as a standard, and recoveries of 95% of theory were obtained.

Decarboxylation of fatty acids was accomplished by treatment with sodium azide as described by Brady et al. (16). The system of Baggiolini and Bickel (17) was used to collect the evolved $^{14}CO_2$ by using ethanolamine: methyl cellosolve (1:2, v/v) as a trapping agent and a gentle flow of nitrogen as carrier gas. After termination of the reaction, 10 ml of toluene scintillant (13) were added to the vial, and radioactivity was determined.

Liver lipids and liver and plasma cholesterol

[2] Extreme care was necessary to prevent losses of the volatile esters of shorter-chain fatty acids; a very gentle nitrogen flow was essential. Temperatures above room temperature and reduced pressures were to be avoided. Losses of up to 50% of the lower-chain fatty acids were observed when these precautions were not carefully adhered to.

TABLE II

Body Weight and Plasma and Liver Lipids of Rats Fed Different Fats

Dietary fat	Final body weight	Plasma		Liver[a]		Plasma-liver cholesterol pool
		Total lipids	Cholesterol	Fat	Cholesterol	
	g	mg/100 ml	mg/100 ml	%	mg/g	mg
			Experiment I[b]			
Corn oil	269 ± 5[c]	408 ± 25	66 ± 2	5.0 ± 0.1	3.65 ± 0.12	50 ± 1
Coconut oil	274 ± 5	339 ± 12	65 ± 2	5.2 ± 0.1	3.61 ± 0.17	52 ± 2
MCT	264 ± 2	249 ± 21	49 ± 2	5.0 ± 0.1	3.21 ± 0.18	42 ± 2
Corn vs coconut[d]	ns	P < .050	ns	ns	ns	ns
Corn vs MCT	ns	P < .001	P < .001	ns	ns	P < .005
Coconut vs MCT	ns	P < .005	P < .001	ns	ns	P < .005
			Experiment II[b]			
Corn oil	257 ± 6[c]	409 ± 28	76 ± 3	5.5 ± 0.3	4.98 ± 0.33	66 ± 5
Coconut oil	259 ± 7	402 ± 40	68 ± 3	5.1 ± 0.1	3.34 ± 0.08	47 ± 2
MCT	244 ± 5	264 ± 15	52 ± 2	4.7 ± 0.1	3.20 ± 0.04	40 ± 1
Corn vs coconut	ns	ns	ns	ns	P < .001	P < .005
Corn vs MCT	ns	P < .001	P < .001	P < .025	P < .001	P < .001
Coconut vs MCT	ns	P < .010	P < .001	P < .050	ns	P < .010
			Experiment III[b]			
Corn oil	263 ± 5[e]	835 ± 62	99 ± 4	5.6 ± 0.2	3.47 ± 0.13	55 ± 2
MCT	254 ± 3	494 ± 54	68 ± 6	5.3 ± 0.2	2.83 ± 0.10	40 ± 2
Corn vs MCT	ns	P < .001	P < .001	ns	P < .001	P < .001

[a] Liver values are expressed on a wet-weight basis.

[b] Experiments I and II were of three-weeks duration and Experiment III, of two-weeks duration. Mean initial body weights for Experiments I, II, and III were 185, 146, and 179 g respectively.

[c] Mean for eight rats ± standard error of the mean.

[d] Probability of the difference being significant; ns = not significant.

[e] Mean for 10 rats ± standard error of the mean. The MCT group contained nine rats.

TABLE III

Body Weight and Plasma and Liver Lipids of Rats Fed Cholesterol-Supplemented Diets Containing Different Fats

Dietary fat	Body weight	Plasma		Liver[a]		Liver-plasma cholesterol pool
		Cholesterol	Total lipids	Fat	Cholesterol	
	g	mg/100 ml	mg/100 ml	%	mg/g	mg
		Experiment IV[b]				
Corn oil	265 ± 3[c]	218 ± 16	915 ± 47	18.7 ± 0.5	20.8 ± 0.6	330 ± 6
Coconut oil	267 ± 2	200 ± 14	878 ± 56	15.3 ± 0.7	15.6 ± 0.3	243 ± 8
MCT	258 ± 6	243 ± 16	1040 ± 54	14.8 ± 0.6	16.2 ± 1.1	235 ± 19
Corn vs coconut[d]	ns	ns	ns	$P < .005$	$P < .001$	$P < .001$
Corn vs MCT	ns	ns	ns	$P < .001$	$P < .005$	$P < .001$
Coconut vs MCT	ns	ns	ns	ns	ns	ns
		Experiment V[b]				
Corn oil	232 ± 6[e]	167 ± 24	556 ± 79	16.0 ± 1.1	16.3 ± 0.9	201 ± 17
Coconut oil	230 ± 2	133 ± 10	505 ± 42	14.1 ± 1.2	11.8 ± 1.0	142 ± 10
MCT	231 ± 4	183 ± 26	761 ± 124	9.4 ± 0.2	9.3 ± 0.4	124 ± 6
Corn vs coconut	ns	ns	ns	ns	$P < .010$	$P < .025$
Corn vs MCT	ns	ns	ns	$P < .001$	$P < .001$	$P < .005$
Coconut vs MCT	ns	ns	ns	$P < .005$	$P < .050$	ns

[a] Liver values are expressed on a wet-weight basis.

[b] Experiments IV and V were of three-weeks duration. Mean initial body weights for Experiments IV and V were 158 and and 216 g respectively. Female rats were used in Experiment V.

[c] Mean for eight rats ± standard error of the mean.

[d] Probability of the differences being significant; ns = not significant.

[e] Mean for seven rats ± standard error of the mean.

were determined as previously described (18, 19). Total serum lipids were determined by using the procedure of Huerga et al. (20). The data were analyzed statistically by means of the "t" test.

RESULTS AND DISCUSSION

Influence of Dietary Lipids on Plasma and Liver Lipids

The effects of the various dietary fats tested on plasma and liver lipids of rats fed cholesterol-free or -supplemented diets are shown in Tables II and III. The data presented in Table II demonstrate lower plasma and liver total lipid and cholesterol levels for rats fed MCT as compared with values for animals fed corn oil or coconut oil. These results are in agreement with the observations of other investigators (1-4). The depression in plasma and liver cholesterol levels induced by MCT is reflected in the plasma-liver cholesterol pool (sum of total plasma + liver cholesterol) (21), a finding also in agreement with the observations of Kritchesvky and Tepper (4). In rats fed cholesterol-cholic acid supplemented diets, MCT or coconut oil, in comparison with corn oil, did not significantly influence plasma cholesterol or total lipid levels (Table III). This lack of effect of MCT is in contrast to the significant decrease in plasma and liver cholesterol levels observed by Kritchevsky and Tepper (4) in rats fed a cholesterol-supplemented diet. It was found that rats fed coconut oil or MCT had lower liver cholesterol and lipid levels and the plasma-liver cholesterol pool was decreased, but in two separate experiments, as shown in Table III, plasma lipids remained unaltered as a consequence of MCT feeding. The reasons for the difference in results between this study and that of Kritchevsky and Tepper (4) are not clear but may relate to the different strain of rat used, the different levels of fat fed (12 vs 20%), or possibly to other differences in experimental procedure.

The results of the two experiments presented in Table III are in general agreement, but some differences are evident. Although the reasons for these discrepancies are not clear, it should be noted, as indicated in Table III, that the starting weights and sex of the animals used in these two experiments were different and consequently could have contributed to the observed differences.

Effect of Ingested Fat on Fatty Acid Composition

Plasma and liver obtained from rats used in Experiments I and III (Table II) were taken for fatty acid analysis, and the findings are presented in Table IV; as can be seen, the results of the two experiments are in good agreement. The most striking difference observed is the much higher level of linoleate 18:2 in plasma and liver lipids of rats fed the corn oil diet. This observation is not startling since corn oil fatty acids contain over 50% linoleate (Table I). The ingestion of linoleate by coco-

TABLE IV
Liver and Plasma Fatty Acid Composition of Rats Fed Different Fats

Fatty acid[a]	Plasma			Liver		
	Dietary fat:corn oil	Coconut oil	MCT	Corn oil	Coconut oil	MCT
			Experiment I			
10:0	1.7 ± 0.5[b]	1.4 ± 0.2	3.9 ± 0.5	0.6 ± 0.2	1.2 ± 0.4	1.2 ± 0.2
12:0	1.2 ± 0.1	10.3 ± 1.4	1.7 ± 0.3	0.4 ± 0.1	2.4 ± 0.8	0.7 ± 0.1
14:0	2.2 ± 0.2	9.1 ± 0.7	4.9 ± 0.6	1.6 ± 0.2	4.7 ± 0.6	2.3 ± 0.2
16:0	15.4 ± 0.5	22.1 ± 0.4	24.7 ± 0.5	17.9 ± 0.4	23.5 ± 0.7	22.4 ± 0.7
16:1	3.3 ± 0.4	5.9 ± 0.2	8.3 ± 0.4	2.6 ± 0.1	4.7 ± 0.4	4.5 ± 0.2
18:0	5.1 ± 0.2	5.8 ± 0.3	6.1 ± 0.3	11.5 ± 0.6	14.1 ± 0.6	16.2 ± 0.5
18:1	13.9 ± 0.6	14.8 ± 0.6	18.1 ± 1.2	12.7 ± 0.5	14.9 ± 0.6	15.0 ± 0.3
18:2	39.6 ± 1.9	18.0 ± 1.0	18.7 ± 0.7	31.4 ± 1.0	13.9 ± 0.5	14.0 ± 0.5
20:4	16.9 ± 1.9	12.5 ± 1.2	12.9 ± 0.8	21.2 ± 0.8	20.5 ± 0.9	23.5 ± 0.8
			Experiment III			
10:0	0.5 ± 0.1[c]	—	1.5 ± 0.6	0.2 ± 0.1	—	0.6 ± 0.1
12:0	0.7 ± 0.1	—	2.1 ± 0.3	0.4 ± 0.1	—	0.4 ± 0.1
14:0	1.6 ± 0.1	—	4.1 ± 0.3	0.8 ± 0.1	—	1.4 ± 0.1
16:0	17.5 ± 0.6	—	28.2 ± 0.8	19.9 ± 0.4	—	26.3 ± 0.6
16:1	3.2 ± 0.2	—	8.6 ± 0.3	2.7 ± 0.1	—	5.0 ± 0.1
18:0	4.7 ± 0.4	—	5.5 ± 0.5	10.5 ± 1.0	—	13.3 ± 0.4
18:1	16.4 ± 0.8	—	20.4 ± 1.6	12.8 ± 0.4	—	15.9 ± 0.5
18:2	43.7 ± 0.9	—	18.4 ± 0.5	30.7 ± 1.3	—	14.6 ± 0.5
20:4	11.8 ± 1.5	—	10.9 ± 1.0	22.3 ± 0.8	—	22.6 ± 0.4

[a] The number preceding the colon represents the chain length of the fatty acid, and the number following the colon, the number of double bonds.

[b] Mean for eight rats ± standard error of the mean. MCT group contained seven rats.

[c] Mean for five rats ± standard error of the mean.

nut oil- and MCT-fed animals was much lower, being restricted to that contained in the 2% corn oil added to all diets. The lower linoleate content of the plasma and liver lipids of rats fed coconut oil or MCT is partially offset by the higher palmitate levels 16:0 noted. The concentration of shorter-chain fatty acids was higher in the plasma and liver of MCT- and coconut oil-fed rats as compared with values for animals fed corn oil. The level of lauric 12:0 and myristic 14:0 acids was particularly high in plasma of coconut oil-fed rats and was also elevated, but to a lesser extent, in the liver lipids of these animals. This too is undoubtedly a reflection of the high levels of these fatty acids in the ingested coconut oil (Table I). Of significance is the absence of high levels of short-chain fatty acids in plasma and liver lipids of MCT-fed rats in spite of the ingestion of large quantities of these fatty acids. This can probably be explained by the differences in the metabolism of shorter-chain fatty acids. MCT fatty acids are adsorbed via the portal system, transported to the liver, and presumably elongated or oxidized (22,23). Consequently one would not expect to find a large increase in plasma MCT fatty acids. However the lack of a marked increase in the level of these short-chain fatty acids in liver lipids suggests that the pathways involved in processing these acids are extremely active in the MCT-fed rat.

It should be noted that the level of unsaturated fatty acids observed in the tissues of rats used in these studies was quite high. Fatty acids in the tissue lipids of animals fed a low fat diet, therefore representative of endogenously synthesized fatty acids, are characterized by a high level of saturated and monounsaturated acids (24,25). The level of unsaturated fatty acids found in tissue lipids in the present studies is probably a reflection of the pre-experimental diet, Purina rat chow, which contains appreciable quantities of unsaturated fatty acids (Table I). That this is the case is exemplified by the high concentration of the unsaturated fatty acids observed in the livers of rats maintained on lab chow (Table I). In spite of this carry-over effect the relative differences observed are meaningful from a qualitative standpoint and depict the direction of change of the various fatty acids in response to the ingestion of the fats tested.

Utilization of Acetate-1-^{14}C by Adipose and Liver Tissue in Vitro

As shown in Table V, the ingestion of coconut oil- or MCT-supplemented diets significantly increased the ability of isolated epididymal adipose tissue to incorporate acetate-1-^{14}C into fatty acids. Liver slices from rats fed corn oil, coconut oil, or MCT converted acetate to fatty acids at a similar rate; however acetate incorporation into cholesterol was depressed by

TABLE V

Acetate-1-^{14}C Utilization by Adipose and Liver Tissue of Rats Fed Different Fats

Dietary fat	Adipose tissue		Liver		
	CO_2	Fatty acid	CO_2	Fatty acid	Cholesterol
	mμmoles incorporated/100 mg tissue/3 hr				
	Experiment I				
Corn oil	434 ± 27[a]	464 ± 48	2138 ± 109	249 ± 35	11.6 ± 1.6
Coconut oil	424 ± 16	805 ± 41	1946 ± 158	253 ± 59	8.7 ± 2.1
MCT	494 ± 26	977 ± 133	1797 ± 129	223 ± 59	4.7 ± 0.7
Corn vs coconut[b]	ns	P < .001	ns	ns	ns
Corn vs MCT	ns	P < .025	ns	ns	P < .C05
Coconut vs MCT	P < .050	ns	ns	ns	ns
	Experiment III				
Corn oil	354 ± 17[c]	550 ± 39	2793 ± 195	414 ± 70	7.1 ± 0.9
MCT	381 ± 24	899 ± 95	2712 ± 205	278 ± 50	4.4 ± 0.9
Corn vs MCT	ns	P < .005	ns	ns	P < .050

[a] Mean for seven rats ± standard error of the mean.

[b] P = Probability that the comparisons made had significantly different values; ns = not significant.

[c] Mean for 10 rats ± standard error of the mean.

MCT ingestion. These results were reproducible, as shown by the experiments reported in Table V, but the data for hepatic fatty acid synthesis do not agree with the observation of Kritchevsky and Tepper (4), who found an enhanced rate of fatty acid synthesis from acetate by liver of rats fed MCT. Perhaps this difference is related to the longer experimental period in the present study (14 or 21 days vs 7). Results on cholesterol synthesis are in accord with the in vivo results reported by Kritchevsky and Tepper (4) and by Reiser et al. (9), demonstrating a depression in hepatic cholesterol synthesis in rats fed MCT. It is tempting to speculate that the lowered plasma and liver cholesterol levels observed in MCT-fed rats are the result of the depressed rate of hepatic cholesterol synthesis. However additional information concerning the degradative pathways of sterols and the turnover of circulating and tissue sterols is required before any definite conclusions can be reached with regard to the mechanism by which MCT depresses plasma and liver cholesterol levels.

Fatty Acid Composition of Adipose Tissue

Since rat adipose tissue, in contrast to liver, is capable of taking up, esterifying, and accumulating short-chain fatty acids (26), it seemed of value to study this tissue in greater detail. Pooled samples of liver and adipose tissue lipids, obtained from the in vitro studies

TABLE VI

The Incorporation of Acetate-1-^{14}C into Various Fatty Acids by Liver and Adipose Tissue of Rats Fed Corn Oil or MCT (Experiment III)

Fatty acid	% Radioactivity				Fatty acids			
	Adipose		Liver		Adipose		Liver	
	Corn oil	MCT	Corn oil	MCT	Corn oil	MCT	Corn oil	MCT
Pre 10:0[a]		4.5	0	0.5	0.1	4.2	0	0.9
10:0	2.9[b]	1.1	0	0	0.2	0.3	1.1	0.4
12:0	1.8	3.0	0	3.3	0.3	2.0	3.6	1.7
14:0	8.2	6.3	5.8	2.7	1.3	3.0	2.7	3.8
15:0	1.8	1.1	0	3.3	0.4	0.8	0	2.6
16:0	54.1	50.7	58.0	45.6	19.5	31.0	20.8	24.7
16:1	7.1	8.6	6.5	8.4	4.3	9.0	5.0	7.7
X[c]	—	—	—	1.6	0.6	—	—	—
18:0		6.3	8.7	14.3	3.1	5.0	8.1	13.2
18:1	15.9[b]	15.3	7.8	11.5	26.1	29.0	15.5	15.4
18:2	2.9	0	6.5	2.7	41.4	13.0	30.6	11.5
18:3	1.2	1.1	0	0	Tr	14.0	0	0
20:0	0.6	0	0	0	1.9	0	0	0
20:4	3.5	1.0	6.5	6.0	0.7	1.0	12.7	17.5

[a] The number preceding the colon indicates the chain length of the fatty acid, and the number following the colon represents the number of double bonds.

[b] Values represent combination of pre C_{10}:0 and C_{10}:0, and C_{18}:0 and C_{18}:1 fatty acids respectively.

[c] Unidentified fatty acid.

with acetate-1-^{14}C in Experiment III, were studied, and the results are shown in Table VI. These data demonstrate that the adipose tissue lipids of MCT-fed rats show little increase in short-chain fatty acids, perhaps reflecting the efficiency of the liver in removing and metabolizing the shorter-chain acids so that very little of the unmodified acids reaches the adipose tissue. The major difference in adipose tissue fatty acid composition between rats fed corn oil and MCT is the lower linoleate and higher palmitate levels in tissue of MCT-fed rats. These differences are in the same direction as observed for liver and plasma lipid fatty acids but are even more marked in adipose tissue. The data in Table VI also show that the pattern of acetate-1-^{14}C incorporation into fatty acids by adipose tissue is not greatly influenced by MCT feeding; both in the tissue of animals fed corn oil or MCT more than 50% of the incorporated radioactivity was found in palmitic acid. Liver slices from MCT-fed rats incorporated less radioactivity from acetate-1-^{14}C into palmitate and more into stearate and oleate than did tissue from corn oil-fed rats, suggesting, as previously proposed (9,10), a greater rate of chain elongation in the liver of MCT-fed animals.

Evaluation of the Chain-Elongation Pathway

The suggestion that MCT ingestion stimulates fatty acid chain-elongating activity was tested in both liver and adipose tissue by determining the relative incorporation of ^{14}C from acetate-1-^{14}C into the carboxyl group of fatty acids. Also the ability of liver to convert palmitate-1-^{14}C to C_{18} fatty acids was determined. These data are presented in Table VII. Liver and adipose tissue fatty acids from MCT-fed rats had a higher percentage of radioactivity in the carboxyl group than did acids from tissues of rats fed corn oil or coconut oil in Experiment I. The magnitude of this difference for adipose tissue was small and could not be reproduced in Experiment III. The liver fatty acids of MCT-fed animals reproducibly showed greater carboxyl radioactivity than acids from rats fed corn oil, demonstrating greater chain-lengthening activity in the livers of MCT-fed animals. This is also suggested by the conversion of palmitate to C_{18} acids; although the difference was not statistically significant because of the large variability within the MCT group, liver slices from MCT-fed rats did convert 28% more palmitate to C_{18} acids than did tissue of animals fed corn oil. These data, when considered with the results in Table VI, permit the tentative conclusion

TABLE VII

An Estimation of Fatty Acid Elongation by Liver and Adipose Tissue of Rats Fed Different Fats

Dietary fat	Decarboxylation of fatty acids synthesized from acetate-1-^{14}C		Conversion of palmitate-1-^{14}C by liver
	Adipose	Liver	
	% of radioactivity in carboxyl carbon		% of radioactivity in C_{18} acids
	Experiment I		
Corn oil	10.0±0.7[a]	19.3±0.8	2.5±0.4
Coconut oil	9.8±0.4	18.3±2.1	2.1±0.2
MCT	12.7±0.3	28.7±2.6	3.2±1.3
Corn vs coconut[b]	ns	ns	ns
Corn vs MCT	P<.005	P<.005	ns
Coconut vs MCT	P<.001	P<.010	ns
	Experiment III		
Corn oil	11.2±0.9[c]	20.3±1.3	
MCT	10.3±0.4	29.6±2.2	
Corn vs MCT[b]	ns	P<.005	

[a] Mean for seven rats ± standard error of the mean.

[b] Probability of the differences being significant; ns = not significant.

[c] Mean for 10 rats ± standard error of the mean.

that chain-lengthening activity is increased in the liver but not in the adipose tissue of rats fed MCT. The data also demonstrate that virtually no chain lengthening occurs in adipose tissue. If an average fatty acid length of 17 carbons is assumed, de novo synthesis of fatty acids from acetate-1-^{14}C should yield 11% of the radioactivity in the carboxyl carbon, a value extremely close to that observed. In liver, on the other hand, the observed values suggest considerable chain-lengthening activity even in the tissue of rats fed a fat containing predominantly long-chain fatty acids, corn oil, and this activity is increased by 44-48% when MCT is substituted for corn oil in the diet.

Fatty Acid Desaturation Activity

The ability of liver slices to desaturate fatty

TABLE VIII

An Estimate of Fatty Acid Desaturation Activity in Liver Slices of Rats Fed Different Fats (Experiment I)

Diet	Stearate-2-^{14}C incorporated into		
	18:0	18:1	18:2
	% of recovered radioactivity		
Corn	80.1±2.2[a]	14.1±1.4	5.7±0.8
Coconut	75.9±1.4	19.5±1.4	4.5±0.4
MCT	69.4±1.2	25.8±1.1	4.8±0.4
Corn vs coconut[b]	ns	P<.025	ns
Corn vs MCT	P<.005	P<.001	ns
Coconut vs MCT	P<.005	P<.005	ns

[a] Mean for seven rats ± standard error of the mean. MCT group contained six rats.

[b] Probability of the differences being significant; ns = not significant.

acids was determined by incubating slices in a buffer containing stearate-2-^{14}C and determining the distribution of radioactivity in gas chromatographic peaks relating to oleate 18:1 and linoleate 18:2. The results of this experiment are shown in Table VIII. Liver slices from rats fed coconut oil or MCT demonstrated an enhanced capacity to desaturate stearic acid to oleic acid as compared with liver from corn oil-fed rats. The conversion of stearate-2-^{14}C to oleate-^{14}C was 38% higher in the liver of rats fed coconut oil and 83% higher in the tissue of animals fed MCT than in the tissue of rats fed corn oil.

The data presented in Table VIII show that liver slices from rats fed corn oil-, MCT-, or coconut oil-supplemented diets were capable of desaturating stearic acid to a C_{18}-diunsaturated acid. This acid was not identified, but it should be stressed that it probably is not linoleic acid. Recently Holloway et al. (27) have shown that rat liver microsomes are capable of desaturating stearic acid to a diunsaturated acid which is different from linoleic ($\triangle^{6,9}$ vs $\triangle^{9,12}$ for linoleate). The 18:2 acid formed from stearate in the present studies undoubtedly is this acid.

The results of these studies corroborate the findings of other investigators with respect to the hypocholesterolemic effects of MCT and extend these observations by demonstrating that hepatic cholesterol synthesis is impaired in rats fed this fat. The data presented also show that the activity of pathways involved in the lengthening and desaturation of fatty acids is greater in the liver of rats fed MCT as compared with animals ingesting corn oil. Adipose tissue of rats fed MCT develops an increased lipogenic capacity, but its chain-lengthening activity does not seem to be altered by the ingestion of MCT.

The observed decrease in hepatic cholesterol synthesis in the tissue of MCT-fed rats suggests that the decreased plasma and liver cholesterol levels may be the result of the decreased synthetic rate. However such a conclusion, though appealing, is not justified by the results obtained. Also the decreased rate of synthesis observed is not explained by the studies reported. It is possible that the increased acetate utilization for fatty acid elongation might divert the acetate from the pathway of cholesterol synthesis. It has been shown that fatty acid and cholesterol synthesis are inversely related under certain circumstances (28), and it is conceivable that such a relationship exists between other acetate-utilizing pathways, such as fatty acid elongation, and cholesterol synthesis.

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The Lipids of the Aging Human Brain[1]

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ABSTRACT

Lipid compositions of the brains of human beings of different ages from 26 to 80 years have been determined by a combination of chromatographic techniques. No significant differences in composition were found among any of the adult age-groups, and it thus appears that the decline in brain weight and function with age is not associated with loss of any particular type of lipid.

INTRODUCTION

THE STUDY OF THE AGING PROCESS may be pursued in a wide variety of fields, ranging from the chemical to the social and from a consideration of those changes associated with the development of the very young to the degenerative changes that become dominant in the very old. Of particular interest to the biochemist is the so-called molecular aging, that is, the irreversible changes occurring with time in molecules that ordinarily have a very low turnover rate. It seems evident that these changes are of paramount importance in the degenerative processes since the molecules involved are necessarily those that insure the continuity of the tissue and the organism.

In this sense the brain is of particular interest since it contains structures which may be permanent throughout the lifetime of the individual (1) and in which the subtlest changes may be reflected in altered behavior patterns. Some studies have already indicated that alterations in the lipid pattern of the brain may occur with aging. The increase in α-hydroxy acids in the brain sphingolipids has already been noted by Kishimoto and Radin (2), and the possible interaction of accumulating lipid peroxides with neighboring proteins has been postulated in many studies.

For these reasons, the lipids of human brains of different age-groups have been analyzed as completely as possible and are compared. Although some analyses have been carried out on human brain lipids, in general these have been individual cases and no attempt has been made to compare the complete lipid spectrum at different ages. One preliminary study which relates normal brain lipid composition to that in certain disease states has appeared (3).

EXPERIMENTAL SECTION

Materials

The solvents used were all ACS reagent grade and were redistilled and de-oxygenated prior to use. Chloroform and 2,2-dimethoxypropane were redistilled immediately before each column run. The nitrogen (Hi-Pure nitrogen) was obtained from Liquid Carbonics, General Dynamics and contained less than five parts per million of oxygen. Florisil was purchased from Floridin Company (60-100 mesh) and was washed with water, dried in vacuum at 100C and stored in chloroform:methanol (2:1). Mallinckrodt silicic acid, 100 mesh ("suitable for chromatographic analysis") was washed with HCl, water, methanol, and chloroform (4), dried in vacuum at 90C and stored in chloroform:methanol (2:1). DEAE cellulose was used in the acetate form after a thorough wash with 1N HCl, water, and 1N KOH. Glass columns (2.5 cm and 4.0 cm I.D.) fitted with Teflon stopcocks were used for chromatography.

The Desaga apparatus for thin-layer chromatography (TLC) (5) was used for the preparation of plates and samples; the samples were spotted by using 10λ disposable pipettes (microcaps from Drummond, Broomall, Pa.). Silica Gel G (Merck) was the adsorbent employed, and spots were detected with a basic bromophenol blue spray (6). Standards of high purity were obtained from Applied Science Laboratories.

Subjects

All analyses were made on whole human brains which were obtained at autopsy and frozen immediately in dry ice. In none of the subjects studied was there evidence of cerebral pathology on gross examination or after sectioning of the cerebral cortex.

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TABLE I

Subject	Sex	Age	Cause of Death
1	M	2 months	Asphyxia
2	F	26 years	Automobile accident
3	F	28 years	Automobile accident
4	M	38 years	Automobile accident
5	M	53 years	Myocardial infarction
6	M	75 years	Bronchopneumonia
7	M	77 years	Myocardial infarction
8	M	80 years	Congestive heart failure

Extraction of Tissues

All specimens were frozen immediately after removal at autopsy and stored at –20C prior to use. Total gray and white matter were separated from whole cerebral hemispheres by careful dissection of the slightly thawed tissue in the cold (4C). Particularly in the older brains, complete separation of gray and white matter was difficult, and some myelin lipids may account for the high total lipid values of the gray matter. The separated tissue was placed in small plastic bags, forming flat packets. Five- or ten-gram portions were sliced from the desired packet for lipid extraction.

Total lipid was extracted with chloroform: methanol (2:1) in a nitrogen atmosphere, as has been described in detail by Rouser et al. (4) and Folch et al. (7). All extraction procedures and subsequent handling of samples were carried out in the cold and in an atmosphere of nitrogen to prevent destruction of the unsaturated fatty acids of the lipids. Lipid fractions not to be analyzed immediately were sealed under nitrogen, dissolved in cyclohexane in small glass ampules, and stored at –20C. The values for the lipid content of each tissue fraction were obtained as described by O'Brien and Sampson (8).

Column Chromatography

The column chromatographic procedures employed for the isolation of lipids from the brain tissue were based on methods of Rouser et al., as described in detail by O'Brien and Sampson (8). The first fraction removed from the DEAE cellulose column with chloroform: methanol (9:1) was freed of solvent by using a Buchler flash evaporator, was desiccated overnight, and was rechromatographed on a silicic acid-silicate-water column (4.5x20cm) with a small amount of coarse clean sand layered over a small glass wool plug at the bottom of the column. Before the column was poured, the adsorbent was suspended in chloroform and the fine material was decanted and discarded. Nitrogen pressure was used to regulate the flow rate of the eluting solvents. The fractions were eluted as follows:

cholesterol	1,000 ml chloroform
ceramide	500 ml chloroform: methanol (9:1)
cerebroside	2,000 ml chloroform: methanol (4:1)
phosphatidyl choline	1,500 ml chloroform: methanol (4:1) + 0.5% water
sphingomyelin	500 ml methanol + 2% water

The columns were continuously monitored by TLC, using one-ml aliquot from alternate 40-ml fractions collected. Each was rapidly evaporated to dryness in a hot water bath under a stream of nitrogen and then plated on Silica Gel G. If overlapping of fractions oc-

TABLE II

Solvent Systems and R_f's of Lipid Components from Column Chromatograms

Solvent System	Lipid	R_f
Chloroform:methanol:ammonium hydroxide 97:3:0.06	Cholesterol	0.8
	Ceramide	0.2
Chloroform:methanol:water[a] 85:12:2.5 (6)	Cerebroside (major spots)	0.7,0.8
	Lecithin	0.3
	Sphingomyelin	0.2
	Lysolecithin	0.0
Chloroform:methanol:ammonium hydroxide:water 85:25:0.5:2.5 (10) (using "weakened" plates) (5)	Phosphatidyl ethanolamine	0.4
	Phosphatidyl serine	0.2
Chloroform:methanol:ammonium hydroxide 80:20:0.4 (9)	Cerebroside	0.5,0.7
	Cerebroside sulfate	0.15,0.19

[a]For this solvent system the plates must be made very active by heating under an infrared lamp or in an oven for five minutes prior to spotting and one minute before developing to effect a clean separation of lecithin and sphingomyelin.

TABLE III
Composition of Human Brain Lipids (% Dry-Weight Tissue)

	Infant	Young				Middle Age				Old					
	2-mo.	26-yr.		28-yr.		38-yr.		53-yr.		75-yr.		77-yr.		80-yr.	
	Total	Gray matter	White matter	Gray matter	White matter	Gray matter	White matter	Gray matter	White matter	Gray matter	White matter	Gray matter	White matter	Gray matter	White matter
Water	90.2	88.2	76.0	85.8	76.5	85.7	75.6	84.8	77.5	86.3	74.5	85.7	75.5	86.4	75.3
Total lipid															
% wet weight	4.6	5.8	17.6	8.0	17.2	7.6	16.5	7.5	15.2	5.6	15.9	6.4	17.0	5.8	16.5
% dry weight	33.6	49.7	73.2	56.8	73.1	49.3	70.4	49.2	67.4	41.2	62.1	43.1	68.7	42.0	66.8
Cholesterol	8.0	8.5	18.0	14.7	16.2	10.4	17.3	9.5	17.3	8.2	15.2	11.4	17.5	9.0	12.7
Phospholipids, total	—	21.1	28.0	25.4	29.9	23.0	30.4	20.2	22.8	17.4	24.3	19.4	29.1	19.0	26.4
Phosphatidyl ethanolamine	9.5	5.6	9.5	9.3	11.4	6.5	10.7	8.4	8.2	7.8	9.3	8.3	10.4	7.1	10.8
Phosphatidyl serine	a	3.5	4.8	3.2	2.2	4.0	4.7	2.1	a	1.8	4.2	3.2	4.3	2.0	3.3
Phosphatidyl choline	13.1	8.6	10.7	8.7	9.5	8.7	10.1	7.8	9.6	6.1	6.3	4.2	9.0	6.6	5.8
Sphingomyelin	0.6	3.4	3.0	4.2	6.8	3.8	4.9	1.9	5.0	1.7	4.5	3.7	5.4	3.3	6.5
Sphingolipids total	2.2	—	20.7	16.6	21.2	13.7	20.7	—	21.2	8.1	19.5	10.0	23.0	10.2	23.0
Ceramide	0.4	0.8	0.7	0.8	0.7	0.3	0.5	0.4	0.7	0.4	0.6	0.3	0.4	0.7	0.4
Cerebroside	1.0	4.6	15.7	8.7	12.6	6.7	14.1	3.1	14.2	4.3	11.9	5.2	15.4	5.7	14.6
Cerebroside sulfate		a	1.3	2.9	1.1	2.9	1.2	a	1.3	1.7	2.5	0.8	1.8	0.5	1.5

[a]Not determined.

curred (as frequently happened with the chloroform:methanol (4:1) + 0.5% water eluent), the mixture was collected, freed of solvent, desiccated overnight, and rechromatographed on a small (2.5x15 cm) silicic acid-silicate-water column for complete separation of the lecithin and sphingomyelin of the mixture. The MeOH fraction from DEAE often exhibited traces of lysolecithin, but it was impossible to separate sphingomyelin and lysolecithin completely by column chromatography. TLC gave an excellent separation.

Thin-Layer Chromatography

The columns were monitored, and the purity of each lipid fraction was checked by TLC on Silica Gel G plates. The solvent systems employed and R_f's of the lipid components are shown in Table II.

Basic Bromophenol blue (3',3",5',5"-tetrabromophenolsulfonephthalein) was used to spray the plates for visualizing the lipid spots (6). To identify phosphatidyl ethanolamine and phosphatidyl serine, ninhydrin in butanol was employed (Ninhydrin Aerosol Bomb, Nutritional Biochemicals Corporation), the plates were heated for five minutes at 100C, and the position of the amino-containing lipids was noted. The plates were then cooled and sprayed with pararosanaline, which stains all lipids. Bromophenol blue will not stain lipids on a plate which has been previously sprayed with ninhydrin.

RESULTS AND DISCUSSION

In Table III are listed the compositions of brain lipids as percentage of dry weight of the tissue. They are arbitrarily grouped into three different age levels: young, middle-aged and old, and similar data from a two-month-old brain have been included for comparison. In general, the values agree with those obtained by other laboratories (8-14), where comparisons are possible.

The salient feature of these results is that there appear to be no significant differences in lipid composition of the brains of different adult age-groups; the differences within each group are of the same order of magnitude as are those between age groups. This means that differences in function, if such occur at these ages, are not reflected in these analyses. Loss of brain lipids with aging therefore appears not to result in changes in composition and is probably a consequence of the loss of whole cells with their complete lipid complement (15). A corollary to these findings would be that significant alterations of the brain lipid patterns may be the result of disease states or other abnormalities.

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The Distribution of Labeled Palmitic Acid into the Diglycerides and Triglycerides of Rat Adipose Tissue

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ABSTRACT

After in vitro incubation of rat epididymal fat pads with radioactive palmitic acid, the distribution of the label in the different lipid classes and in different triglycerides was determined by silica gel and silver nitrate-silica gel thin-layer chromatography (TLC).

The radioactivity of the diglycerides was approximately half of the triglycerides. This ratio did not change with alteration in the time of incubation. It remained unaltered even after a subsequent 10-min incubation in a nonradioactive medium.

When the fat pads were incubated, first with ^{14}C-, then with ^{3}H-labeled palmitic acid, the $^{3}H/^{14}C$ ratio was slightly lower in diglycerides than in triglycerides.

The fully saturated molecules contained 38% of the radioactivity of triglycerides. Addition of oleic acid or norepinephrine to the labeled palmitic acid-containing medium decreased this value. Subsequent incubation with these compounds did not alter the distribution of radioactivity.

INTRODUCTION

A NUMBER OF INVESTIGATIONS of the incorporation of radioactive labeled fatty acids into the lipids of different tissues and tissue preparations show that significant radioactivity is found in diglycerides. For example, incubation of rabbit and dog aortic homogenates shows that respectively 39 and 29% of the total radioactivity of neutral lipids is present in diglycerides(1); that mitochondrial preparations of rat liver incorporate radioactivity into diglycerides to about one-third the extent of that of triglycerides (2); that homogenates of rat epididymal fat pad incorporate half of the total radioactivity into the diglyceride fraction (3); and that in vivo incubation of epididymal fat pads of rats with radioactive labeled fatty acids results in the incorporation of 20% of the total radioactivity into diglycerides (4). The data quoted were by-products of investigations carried out for other reasons. In these studies the origin of the radioactive diglycerides was not discussed, or else the diglycerides were interpreted simply as being precursors of triglycerides.

In the present paper the origin of radioactive diglycerides formed during in vivo incubation of rat epididymal fat pads with radioactive labeled fatty acids is investigated.

MATERIALS AND METHODS

Male Wistar rats (150-200 g) fed ad libitum on Purina chow diet were killed by a blow on the head. The distal part (150-250 mg) of epididymal fat pad was immediately excised, weighed and incubated in a 5-ml medium at 37C with gentle shaking for the time indicated in the tables.

The incubation medium was a Krebs-Ringer phosphate buffer (Ca^{++} omitted; pH 7.4; and 5mM glucose). It contained 5% human serum albumin. The following fatty acids were complexed to the albumin in the different experiments: a) 1-^{14}C-palmitic acid (New England Nuclear Corporation, spec. act. 10 mc/mmol) was used in the experiments reported in Tables I and II; b) 9, 10-$^{3}H_2$-palmitic acid (Radiochemical Center, Amersham, Buckinghamshire, England, spec. act. 256 mc/mmol) was used in the experiments reported in Table II; c) 1-^{14}C-palmitic acid (Reanal, Budapest, spec. act. 1 mc/mmol) was used in the experiments reported in Tables III and IV.

After incubation the tissue was quickly washed in saline and homogenized in alcohol-acetone 1:1 in a glass Potter hand-homogenizer. The homogenate was extracted by shaking for 20 min with 25 ml of this mixture and filtered. One part of the filtrate served for determination of the total radioactivity, and the other part was chromatographed on silica gel plates (thickness 0.3 mm). The developing solvent mixture was petroleum ether (bp to 70C): diethyl ether: acetic acid (70:30:1). Rhodamine B was used as the detecting agent. In the experiments reported in Tables III and IV the triglycerides separated by the TLC technique were applied to silver nitrate-silica gel plates (thickness 0.3 mm) in order to

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TABLE I
Incorporation of 1-^{14}C-Palmitic Acid into Different Lipid Classes by Rat Epididymal Adipose Tissue [a]

Time in minutes	Percentage of total radioactivity			
	2.5	5	10	10 in radioactive medium followed by 10 in fatty acid free medium
	%	%	%	%
Triglycerides	57.1 ± 2.5	63.1 ± 5.4	61.0 ± 0.7	74.7 ± 3.6
Diglycerides	29.6 ± 2.6	25.6 ± 3.8	27.6 ± 1.5	23.4 ± 3.5
Free fatty acids	12.4 ± 3.3	10.3 ± 1.8	10.5 ± 1.9	1.3 ± 0.2
Other lipids [b]	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.1	0.6 ± 0.1

[a] The values reported are means of three animals + S. E. of the mean.
[b] Other lipids: monoglycerides, phospholipids, cholesterol esters, etc.

separate the molecular species according to the number of double bonds (5). The developing solvent in this case was benzene alone. The separated lipids in both chromatographic procedures were scraped off and extracted with diethyl ether for 2 hr in a microsoxhlet apparatus. The radioactivity measurements were carried out by using a Packard-Ticarb scintillation spectrometer.

RESULTS

The results obtained by incubating adipose tissue for different times in a medium containing 1-^{14}C-palmitic acid (0.25 μmol/ml) are summarized in Table I. The total radioactivity taken up by the tissues increased linearly for the time intervals (4). If it is supposed that the radioactivity of the diglyceride fraction represents the radioactivity of those diglycerides originating directly from phosphatidic acid, *i.e.*, the precursor diglyceride in the triglyceride synthesis, then it might be expected that the percentage distribution of the radioactivity would in time be shifted in favor of the triglycerides. The results presented in Table I do not support this hypothesis. The percentage of radioactivity in the diglycerides did not change significantly even when the tissue was incubated for a second 10-min interval in a fatty acid free medium, following a 10-min incubation in the radioactive medium. This is shown in the last column of Table I.

TABLE II
Ratio of 9, 10-$^{3}H_2$-Palmitic Acid to 1-^{14}C-Palmitic Acid in the Different Lipid Classes Isolated from Rat Epididymal Fat Pads [a]

Lipid class	Molar ratio ^{3}H:^{14}C
Triglycerides	1.07 ± 0.06
Diglycerides	0.88 ± 0.05
Free fatty acids	4.14 ± 0.90
Others [b]	1.29 ± 0.12

[a] The values reported are the means of four animals ± S. E. of the mean.
[b] Others: monoglycerides, phospholipids, cholesterol esters, etc.

In this case, naturally, the percentage of radioactivity of the free acids fell to very low levels.

In order to clarify these relationships further, the following experiment was carried out. Adipose tissue was incubated first in medium containing 1-^{14}C-palmitic acid for 10 min, rinsed, then incubated in a medium containing 9, 10-^{3}H-palmitic acid. The concentration of both acids was 0.25 μmol/ml. The molar ratio of the incorporation of the differently labeled palmitic acid preparations into the different lipids was determined (Table II). The distribution of the two labels between the precursors and the end products is different and time-dependent. Therefore the isotope present in the first medium will be present in a higher proportion in the end product than the isotope of the second medium. In an A → B → C → D → . . . → reaction sequence, the ratio between the incorporation of the second and the first isotope should be higher, the more the compound lies to the left, i.e., the precursors. This ratio is slightly higher in the triglyceride than in the diglyceride (Table II, $p = 0.05$) which suggests a) that the bulk of radioactive diglycerides is not the precursor of the triglycerides and b) that perhaps the radioactive diglyceride is the product of a rapid hydrolysis of newly synthesized triglycerides. However the results reported above can be interpreted by two additional theories: the diglycerides and the triglycerides are in a dynamic equilibrium, or a part of the diglycerides is not immediately transformed to triglycerides but enters a pool with a low turnover rate.

To differentiate between these theories, fat pads were incubated in three different ways. In Group A the fat pads of five rats were incubated individually for 5 min in 1-^{14}C-palmitic acid medium (2 μmol/ml). In Group B the fat pads of three animals were incubated individually for 5 min in a medium containing

TABLE III
Effect of Unlabeled Oleic Acid Incubated with or after ^{14}C Palmitic Acid on the Distribution of Radioactivity in Triglycerides [a]

Number of double bonds in the triglyceride molecule	Percentage of total radioactivity		
	Group A	Group B	Group C
	%	%	%
0	37.7 ± 2.1 [b]	9.1 ± 0.7	38.4 ± 1.2
1	34.8 ± 1.6	36.1 ± 0.6	36.0 ± 1.4
2	17.6 ± 1.0	38.8 ± 2.2	16.3 ± 1.5
>2	9.9 ± 0.6	16.0 ± 1.7	9.3 ± 1.1

[a] Experimental details are described in the text.
[b] Mean value ± S. E. of the mean.

both 1-^{14}C-palmitic acid (2 μmol/ml) and unlabeled oleic acid (4 μmol/ml). In Group C the fat pads of four rats were incubated individually in two different media; first with 1-^{14}C-palmitic acid (2 μmol/ml) for 5 min, quickly washed in saline, then transferred into a second medium containing unlabeled oleic acid (4 μmol/ml) for a 10-min incubation.

The distribution of the radioactive label in the different triglycerides is a function of the mixing of the labeled saturated acids with the unlabeled unsaturated acids in the course of the triglyceride synthesis. In Group A, as Table III shows, not all of the radioactivity is present in the trisaturated triglycerides because it is mixed with endogenous tissue fatty acids. In Group B the oleic acid and labeled palmitic acid mixture caused a significant decrease of the radioactivity of the completely saturated triglycerides. According to the dynamic equilibrium hypothesis (Group C), both the labeled tripalmitic and unlabeled trioleic acids should be degraded and resynthesized. This process should result in an exchange of fatty acids which will diminish the radioactivity of the completely saturated triglycerides. In contrast to this hypothesis, the subsequent incubation with oleic acid had no effect at all upon the radioactivity of the triglycerides.

Similar results were obtained if, instead of adding oleic acid into the medium, the free fatty acid level in the adipose tissue was increased by norepinephrine. In this experiment, summarized in Table IV, the medium of Group A contained only 1-^{14}C-palmitic acid (2 μmol/ml), and the incubation period was 10 min. In Group B norepinephrine (2 μg/ml) was added to the 1-^{14}C-palmitic acid medium, and the incubation period was 10 min. Group C of fat pads was incubated first with labeled palmitic acid (2 μmol/ml) for 10 min, washed, then transferred to a second medium containing norepinephrine (2 μg/ml) but no exogenous fatty acids. The changes are again significant in Group B but not in Group C (Table IV).

DISCUSSION

The reaction sequence of triglyceride synthesis according to Weiss et al (6) follows. The (alpha)-glycerophosphate forms phosphatidic acid with the addition of two fatty acids. Phosphate is split off of the phosphatidic acid to yield the diglycerides. In the last step the free hydroxyl-group of the diglyceride is esterified. In the present study the labeled palmitic acid behaved as a normal precursor, that is, it disappeared from the free fatty acid fraction and was incorporated into diglycerides and triglycerides. The turnover rate of the phosphatidic acid must be high because it never showed significant radioactivity. In contrast to the phosphatidic acid, the radioactivity of the labeled diglycerides remained about half that of the triglycerides, independent from the time of incubation. This constant ratio could be explained by a dynamic equilibrium of the

TABLE IV
Effect of Norepinephrine Incubated With or After 1-^{14}C-Palmitic Acid on the Distribution of Radioactivity in the Triglycerides [a]

Number of double bonds in the triglyceride molecule	Percentage of total radioactivity		
	Group A	Group B	Group C
	%	%	%
0	37.5 ± 1.8 [b]	16.1 ± 4.6	33.4 ± 1.6
1	34.3 ± 0.1	31.5 ± 1.3	34.6 ± 0.6
2	16.8 ± 0.9	30.7 ± 0.1	18.3 ± 2.2
>2	11.3 ± 2.8	21.7 ± 0.1	13.7 ± 1.6

[a] Experimental details are described in the text.
[b] The values reported are the means of 3 animals ± S. E. of the mean.

diglycerides and triglycerides; however the experiments presented in Tables III and IV speak against this hypothesis. It is possible that a part of the labeled diglycerides enter a second fat cell diglyceride pool with a low turnover rate. Recently published data (7, 8) indicate, besides the de novo triglyceride synthesis via (alpha)-glycerophosphate, the existence of another mechanism for the incorporation of fatty acids into triglycerides. It can be assumed that the presence of the two diglyceride pools, showing different turnovers, is connected with the two different modes of triglyceride synthesis.

If a random distribution of the fatty acids in the glycerides is assumed, the proportion of the radioactivity in triglycerides can be calculated in the following way: s = % of saturated fatty acids in the Free Fatty Acid (FFA) pool of triglyceride synthesis; u = % of unsaturated fatty acids in the FFA pool of triglyceride synthesis; T = the radioactivity of the totally saturated triglycerides as % of the total triglyceride radioactivity. Then $(s + u)^3 = s^3 + 3s^2u + 3su^2 + u^3$. The members of the polynomial are proportional to the quantity of the different triglycerides. Their radioactivity shows the following relation since only the saturated fatty acid is radioactive: $3 \times 1s^3$, $2 \times 3s^2u$, $1 \times 3su^2$, $0 \times 1u^3$

$$T = 3s^3 \times 100/3s^3 + 6s^2u + 3su^2 = 100s^2/s^2 + 2su + u^2 = 100s^2/(s + u)^2$$

According to the definition, $s + u = 100$. Therefore

$$T = s^2/100 \qquad \text{and} \quad s = 10\sqrt{T}$$

The total amount of the free fatty acids of the adipose tissue is much higher than that quantity taken up by the tissue from the medium. If the labeled palmitic acid had been mixed with the total amount of the tissue free fatty acids, its distribution in the triglycerides would be determined by the composition of these acids. In this case it would be expected about 10% of the radioactivity would be present in the fully saturated triglycerides. The first columns of Table III and Table IV show that this value is 38% when only saturated acid was added to the medium. This indicates that the fatty acids added to the medium are incorporated into glycerides before they could be mixed with the total amount of the tissue free fatty acids. Table III shows that, for Group B, the s:u ratio was 1:2 in the medium corresponding to that of adipose tissue. Here 10% of the radioactivity was found in the trisaturated triglycerides, which is in good agreement with the formula and the random distribution theory.

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Phospholipase Activity of Rat Tissues and Its Modification by Trypsin[1,2]

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ABSTRACT

Treatment with proteolytic enzymes before the addition of the phospholipid substrate increases the activity of the phospholipases of the spleen, thymus, bone marrow, lung, and liver of the rat. In contrast, the phospholipase activity of the intestine, which is higher than that of all other normal tissues, is not increased when incubated with proteases. The results of fractionation studies by high-speed centrifugation and gel filtration and differences in enzyme kinetics support the conclusion that the intestinal phospholipases differ substantially from phospholipases found in the other tissues.

INTRODUCTION

PREVIOUS REPORTS FROM THIS LABORATORY have shown that the phospholipase activities of rat intestinal mucosa which are greatly reduced after whole body x-irradiation can be augmented by treatment with proteolytic enzymes or a fraction from normal rat mucosa (1-5). A detailed study of the conditions for trypsin activation and the results of experiments with other tissues from normal rats are reported.[3]

MATERIALS AND METHODS

Preparation of Tissue Homogenates and Fractions

Male Osborne-Mendel rats (175-200 g) were killed by cervical dislocation, followed by decapitation. Tissue samples were homogenized in 10 to 20 volumes of cold 12.5 or 25% glycerol, containing $2x10^{-3}$ M cysteamine, $2x10^{-3}$ M ethylenediaminetetracetic acid (EDTA), and $5x10^{-3}$ M $MgCl_2$, then were buffered at pH 6.4 with potassium phosphate (ionic strength $1.5x0.1\mu$). Variations in glycerol concentrations from 12.5% to 25% were introduced for technical reasons during gradient centrifugation studies or column chromatography and had no effect on the activity or distribution of the phospholipases. Routinely the tissue homogenates were cleared by centrifugation at low speed (12,000x*g* for 10 min), followed by separation at high speed (100,000x*g* for 60 min) prior to testing and activation.

Homogenates from normal rat mucosa were the source of the mucosal "activating fraction." The latter was obtained from homogenates in the 25% glycerol medium, centrifuged for 10 min at 12,000x*g* and further separated at 100,000x*g* for 60 min. A 7- to 10-ml aliquot of the final supernatant fraction was chromatographed on DEAE-Sephadex previously equilibrated with 12.5% glycerol containing cysteamine, EDTA, etc., as indicated above. The same medium was employed as eluant. An approximate column height of 20-25 cm with a column diameter of 2.5 cm and an adjusted flow rate between 0.5 and 1.0 ml per minute were used. All operations were carried out at 0.5C. The breakthrough peak, consisting of a light red fraction that contained about 10% of the total protein of the original preparation, possesses the ability to activate the phospholipases of various tissues of the normal rat and of intestinal mucosa of irradiated rats and is the fraction referred to as "activating factor from normal mucosa." This fraction does not exhibit any lecithinase or lysolecithinase activity with the testing procedures.

Estimation of Phospholipase Activity

Active Phospholipases. The methods already described for lecithin preparation and lecithinase assay (6) have been followed in these experiments. Hydrolysis of the phospholipid has been routinely determined by estimating the released fatty acid or the decrease in esterified fatty acids. Lysolecithinase was estimated with

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[2]Presented in part at the AOCS Meeting, Houston, April 1965.

[3]The expressions "activation" and "activators" are operational terms to indicate the process and the agents which can increase the enzymatic activity. Consistent with previous use, the comprehensive term lecithinase has been employed to designate the activities which hydrolyze lecithin with the liberation of two moles of fatty acid and one mole of glycerylphosphorylcholine without the accumulation of measurable amounts of lysolecithin (6,7). The term lysolecithinase indicates the enzyme which hydrolyzes lysolecithin to one mole of fatty acid and one mole of glycerylphosphorylcholine (EC.3.1.1.5.). Alternative designations for this activity are phospholipase B and lysophospholipase.

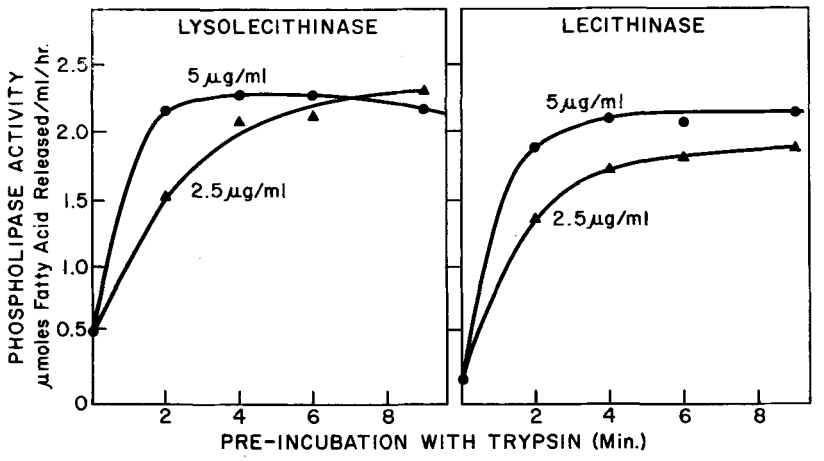

Fig. 1. Effect of preincubation with trypsin on the phospholipases of intestinal mucosa of x-irradiated rats. Mucosal supernatant (100,000xg for one hour from a 1/20 homogenate) was diluted with 4 volumes of buffer containing trypsin at 5 and 2.5 μg/ml final for preincubation at 37C. At selected time-intervals aliquots of 0.5 ml and 0.125 ml were used for assay of lecithinase and lysolecithinase respectively: final reaction volume, 1.5 ml; activity given as μmoles fatty acid released per ml of reaction mixture, per hour.

identical procedure on tissue aliquots one-third to one-fourth the size of the sample used for lecithinase. Lysolecithin, prepared according to Long and Penny (8) and stored frozen at 20 μmoles/ml in water, served as a substrate. Unless otherwise indicated, the final lysolecithin concentration in the reaction mixture was 6.66 μM/ml.

Activation Procedure. The procedure finally adopted for detection and measurement of inactive phospholipases involves the addition of one volume of cold tissue preparation to one volume of buffer or trypsin-buffer at 37C followed, 4 min later, by one volume of substrate at 37C and routine determination of phospholipid hydrolysis. The short incubation time is designed to minimize the effect of temperature on the phospholipases. The use of at least two trypsin concentrations is recommended for all tissues. Those with low protein content (thymus, diluted irradiated mucosa) as a rule are fully activated by low trypsin concentrations, 3 and 10 μg per ml of preincubated mixture. For spleen, lung, bone marrow, and liver usually tested at 1/10-1/20 homogenates, higher trypsin levels are necessary (4,12,20 μg per ml of preincubated mixture). The experimental results leading to this procedure are given in detail in the following section.

Trypsin (2x crystallized, salt-free), α-Chymotrypsin (3x crystallized), and Ovomucoid were purchased from Worthington Biochemical Corporation.

Irradiation. Male Osborne-Mendel rats of 175 to 200 g were irradiated in a Lucite box with an industrial x-ray unit operated at 250 kv and 15 ma. Intrinsic filtration HVL, 0.26 mm Cu equivalent; added filtration, 2 mm Al and 0.25 mm Cu. Target distance, 78 cm; rate, 40 r/min; total dose, 800 r. Controls and irradiated animals were starved from the night preceding the irradiation until killed 24 hr after exposure.

RESULTS AND DISCUSSION

Action of Trypsin on the Mucosa of X-Irradiated Rats

Treatment of high-speed supernatants of mucosa from x-irradiated rats with various proteolytic enzymes (ficin, papain, bromelain, chymotrypsin, and trypsin) results in enhancement of phospholipase activity. Trypsin was selected for the experiments because of its high activity and the availability of specific inhibitors.

The general relationship between the amount of added trypsin, the time of preincubation before the addition of lecithin or lysolecithin and phospholipase activation is shown in Figure 1. In this experiment the mucosal preparation is treated with two concentrations of trypsin, and samples are withdrawn at regular time-intervals and tested for lysolecithinase and lecithinase activity. It is clear that the rate of activation is directly dependent on the concentration of the trypsin and that the same end-point is reached after suitably long periods of preincubation.

TABLE I

Effect of Trypsin on the Lecithinase and Lysolecithinase Activities of Rat Tissues[a]

Tissue	Lecithinase		Lysolecithinase	
	Untreated	Trypsin-Treated	Untreated	Trypsin-Treated
Spleen	24	49	170	298
Lung	19	38	179	265
Thymus	10	44	126	248
Liver	6	10	34	63
Bone marrow	36	77	347	563

[a] High-speed supernatants of 1 to 10 - 1 to 20 (w/v) tissue homogenates were used for all tissues except bone marrow, which was tested as unfractionated homogenate. Tissue aliquots were preincubated for 4 min at 37C with or without trypsin prior to the addition of substrates and three levels of trypsin (4,12,20 μg/ml) were used to ensure maximal activation. Phospholipase activity was determined by estimation of the released fatty acids, then computed to represent the hydrolytic activity of one gram (wet weight) of tissue per hour. The values in the table are the average activity in organs from six rats.

Additional evidence regarding the role of the proteolytic enzymes in the activation process can be summarized as follows. Ovomucoid, which is a trypsin inhibitor, suppresses the activation of phospholipases only when added before the trypsin and is without effect if added at the end of the preincubation period. Once the substrate has been added to samples only partially activated, the initial rate of fatty acid liberation is linear and is not increased by the further addition of chymotrypsin or trypsin. DFP chymotrypsin and alkali- and heat-denaturated trypsin have no activating effect. These findings support the conclusion that trypsin activates the phospholipases without participating in the ensuing hydrolysis of the phospholipid and that the proteolytic action of the enzyme is essential to the activation process.

Similar results were obtained with the same preparation of mucosa of Figure 1 by using the activator of normal mucosa in place of trypsin. It is likely that the same mechanism of activation is involved although a firm conclusion on this point must await purification and identification of the mucosal factor.

Activation and Characterization of Rat Tissue Phospholipases

A number of tissues from normal rats have been tested for phospholipase activities before and after treatment with trypsin. The distribution of activity reported by others (9-12) in untreated homogenates of spleen, lung, and liver has been confirmed. In addition, these data show that the proteolytic enzymes enhance the activity of the phospholipases of all these tissues (Table I).

When homogenates of tissues which contain trypsin-activated phospholipases were submitted to centrifugal fractionation, 80% or more of the phospholipase activity measurable with or without trypsin treatment was found in the high-speed supernatant after four hours at 120,000x*g*. On the other hand, the enzymes from normal rat intestine, which are not affected by trypsin treatment, are sedimented by centrifugation at high speed (6). The difference in sedimentation behavior suggests association with molecular aggregates of different size, and this could be confirmed by gel filtration on Sephadex-G 200. As shown in Figure 2, almost all the activity present in a supernatant of normal mucosa after centrifugation at 120,000 for one hour was recovered in the first effluent from the column: only a minor component appeared in a later fraction.

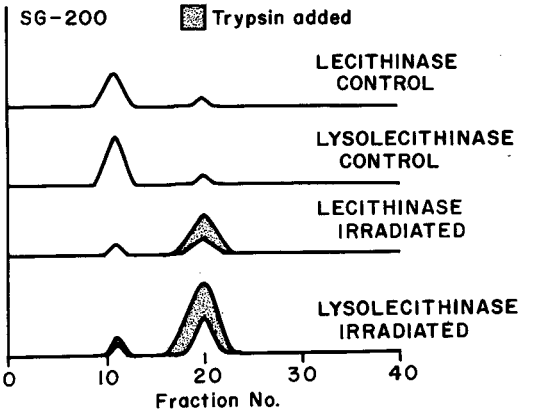

Fig. 2. Elution patterns of high-speed supernatants of mucosa of normal and x-irradiated rats from a Sephadex G-200 column. [Column size: height 29 cm, diameter 2.5 cm. Tissue sample: 3 ml of supernatants (100,000x*g*) from 1/12 mucosal homogenates. Flow rate: 8 ml per hour. Fraction volume: 5 ml.] Phospholipase activity is represented on the ordinate in arbitrary units. The height of the peaks is proportional to the activity of the eluted samples. The shaded areas indicate the increase in activity after preincubation with trypsin. No increase is observed in the samples from the control animal.

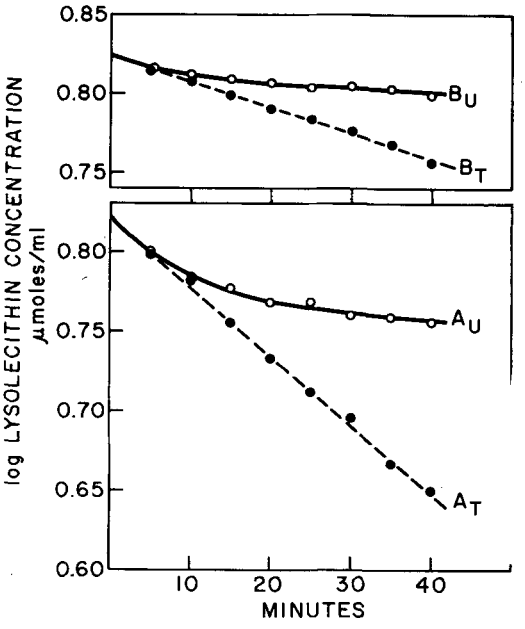

Fig. 3. Kinetics of lysolecithin hydrolysis by irradiated mucosa after treatment with trypsin. Two dilutions (A = 1/10 – B = 1/20) of a high-speed supernatant of mucosa from an irradiated rat were preincubated for 4 min at 37C with (A_T, B_T) and without (A_U, B_U) trypsin (4 μg/ml), then tested for lysolecithinase activity. In the graph the logarithm of the concentration of unhydrolyzed substrate is plotted against time for the two mucosal dilutions used: A in the lower part and B in the upper part of the figure.

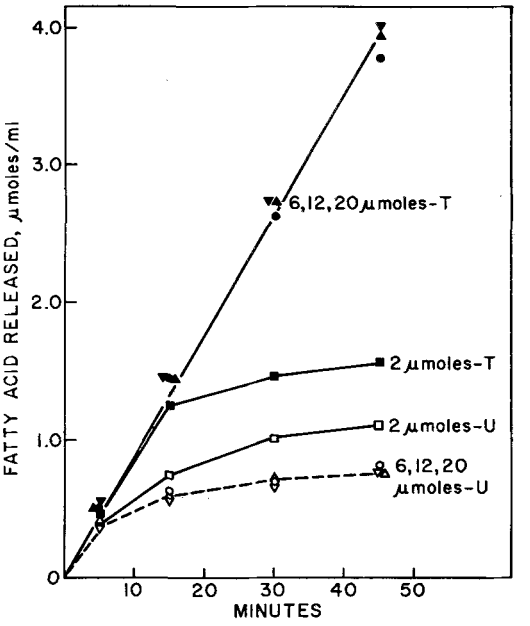

Fig. 4. Effect of variations in substrate concentration and of treatment with trypsin on the lysolecithinase activity of thymus. Aliquots of the high-speed supernatant from a 1/20 thymus homogenate were preincubated with or without trypsin (5μg/ml) at 20C for 6 min, then added with different lysolecithin concentrations for lysolecithinase activity determination. Final lysolecithin concentrations per ml of reaction mixture are indicated in the figure by the following symbols: x = 20 μmoles; △ ▲ = 12 μmoles; ○ ● = 6 μmoles; □ ■ = 2 μmoles. Solid symbols = preincubated with trypsin. Open symbols = preincubated without trypsin.

In the irradiated animal the distribution was completely reversed, and the major portion of the activity (90%) was recovered in the late effluent. In addition, as indicated by the shaded areas in the figure, treatment with trypsin enhanced the phospholipase activity (lecithinase and lysolecithinase) in the irradiated animal but not in the normal. By the use of column markers a preliminary indication of a molecular weight around 50,000 has been obtained for the late peak of the irradiated mucosa. Similar data have been obtained with thymus and spleen supernatants.

These data distinguish two types of phospholipases of rat tissues: a) soluble and trypsin-activated and b) particle-bound and unaffected by trypsin treatment. The first form of the enzyme is found in many tissues, thymus, spleen, lung, bone marrow, liver of the normal rat, and the intestine of x-irradiated rats. The second form has been found thus far only in the intestinal mucosa of normal rats (6,7).

The Effect of Enzyme "Activation" on Enzyme-Substrate Interaction

Activation of the phospholipases involves marked changes in their reactivity with their substrates. For the lecithinase the over-all effect can be simply seen as an increase in activity. The ratio of activities of the untreated and treated preparations is constant at different reaction times and for different enzyme concentrations. First-order kinetics are approximated in both preparations, and the Km values for the untreated and treated samples are identical (1.7×10^{-3} M).

For the lysolecithinase the interaction is more complex. The untreated preparation attacks the lysolecithin but with a progressively falling rate of hydrolysis. Treatment with activators prevents the decline in the rate of the reaction, which now proceeds according to first-order kinetics (Figure 3, A_U and A_T). It

can be shown that a) decreasing the enzyme concentration by one-half decreases product accumulation but does not delay the beginning of deviation from first-order kinetics (compare A_U and B_U of Figure 3); b) reducing the lysolecithin concentration from 20 to 2 μmoles per ml retards the fall in reaction rate so that more phospholipid is hydrolyzed at the lower substrate concentrations (Figure 4). A relatively slow process of enzyme inhibition by secondary interaction with the substrate is indicated by these findings in agreement with the inhibitory effect of high substrate concentrations on lysolecithinases of liver and pancreas reported by others (13,14). It must be emphasized that these findings and considerations do not apply to the intestinal mucosa of normal rats where the enzymes are not activated by trypsin and do not deviate from first-order kinetics.

In sum, this study has shown the presence in rat tissues of two types of phospholipases distinguishable on the basis of distribution, response to treatment with trypsin, sedimentation characteristics, and kinetic behavior. The action of trypsin and the other proteolytic enzymes on the phospholipases is a relatively new finding, and its meaning is still obscure. A true activation, as it occurs for the proteases in general, might be involved. Conversely the removal of a slowly reacting binding site which would eventually cause inhibition could explain the effect on the lysolecithinase.

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The Biosynthesis of Cyclopropane and Cyclopropene Fatty Acids in Plant Tissues

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ABSTRACT

The biosynthesis of cyclopropane and cyclopropene fatty acids was investigated in seeds of several species of the order Malvales, including species with a high content of sterculic acid, a high content of malvalic acid, and a low content of these cyclopropene fatty acids. The fatty acid composition of the lipids in young and developing seeds is compared with particular attention to variations in cyclopropane and cyclopropene fatty acid contents.

Incubation studies employing several ^{14}C compounds indicate that the methyl group of methionine is the most likely precursor of the ring-methylene carbon. A pathway for the synthesis of the cyclopropane and cyclopropene fatty acids is postulated. The origin of malvalic acid is also considered.

INTRODUCTION

THEORIES ON THE BIOSYNTHESIS of the cyclopropene fatty acids can be summarized as follows:

oleic acid + $\bullet CH_2$ → dihydrosterculic acid → dihydromalvalic acid
↓ ↓
stearolic acid + $\bullet CH_2$ → sterculic acid → malvalic acid
↑
C_{17} monounsaturated acid + $\bullet CH_2$ → dihydromalvalic acid

Investigations carried out in this laboratory on the biosynthesis of cyclopropene fatty acids have been briefly reported in a review (1) of the chemistry and biological effects of the cyclopropene compounds. These investigations showed that the incubation of slices of immature *Malva parviflora* fruit with L-methionine [^{14}C-methyl] led to the labeling of dihydrosterculic acid with only minor amounts of labeled malvalic or sterculic acids. Of the several compounds tested, methionine was the most specific precursor of dihydrosterculic acid.

The bacterial cyclopropane fatty acid, lactobacillic acid, is produced by the addition of the methylene group derived from the methyl group of S-adenosyl methionine across the double bond of *cis*-vaccenic acid attached to phosphatidyl ethanolamine (2,3,4). The biosynthesis of the bacterial cyclopropane fatty acids has recently been reviewed (5).

Recently Hooper and Law (6) showed that the methyl group of L-methionine was a precursor of the methylene group of cyclopropane and cyclopropene fatty acids in young seedlings of *Hibiscus syriacus*. They showed that dihydrosterculic acid represented 40% of the labeled fatty acids produced by the seedlings. The cyclopropene fatty acids contained only a small amount of the label, and these authors were unable to determine whether or not dihydrosterculic acid was the precursor of the cyclopropene fatty acids.

From the labeling pattern of sterculic and malvalic acids isolated after the incubation of young *H. syriacus* seedlings with 1-^{14}C-acetate, Smith and Bu'Lock (7) showed that the label was not incorporated into the methylene carbon of the ring. They suggested that sterculic acid was formed by alkylation of octadec-9-ynoic (stearolic) acid and that the formation of malvalic acid must involve chain shortening of some precursor fatty acid.

Kircher and Heywang (8) showed that eggs from hens fed one gram of methyl dihydrosterculate daily developed pink whites during storage. This observation led them to suggest that the hen is capable of desaturating dihydrosterculic acid to produce sterculic acid, a causal factor of this egg disorder. By analogy, they suggested that the desaturation of dihydromalvalic and dihydrosterculic acids in plants could lead to the formation of malvalic and sterculic acids, a suggestion which has also been made by Wilson, Smith, and Mikolajczak (9).

In lipids containing both cyclopropane and cyclopropene fatty acids, methods have not hitherto been available for the separate estimation of sterculic, dihydrosterculic, malvalic,

dihydromalvalic acids and their homologues. Such a method has been described in a separate paper (10) and applied in this investigation.

In this communication, studies on the biosynthesis of cyclopropane and cyclopropene fatty acids in tissue slices of seeds and fruits of the order Malvales are reported, and changes in total lipid content and in cyclopropane and cyclopropene fatty acid composition of triglycerides and polar lipids are discussed in relation to seed maturity. Since the major cyclopropene fatty acid in *Sterculia foetida* is sterculic acid and that of *Brachychiton acerifolium* and *B. populneum* is malvalic acid, studies with these species were conducted to determine whether there was a different route for synthesis of malvalic and sterculic acids. This biosynthesis was also investigated in plants with a low content of cyclopropene fatty acids, namely, the commercially important member of the order Malvales, *Gossypium hirsutum* (cotton), and in some *Malva* species.

EXPERIMENTAL

Materials

Seeds or fruits from the following plant species were used: family Malvaceae—*Malva parviflora* L., *Malva nicaensis* All., *Malvaviscus arboreus* Cav., var. mexicanus Schlecht, *Gossypium hirsutum*, var. empire; family Sterculiaceae—*Sterculia foetida* L., *Brachychiton acerifolium* F. Muell., *Brachychiton populneum* (Schott) R.Br.

As it was rarely possible to determine their precise age, the seeds were arbitrarily classified as young or mature, according to the following criteria. The young seeds were those taken from fruits that had attained approximately half their fully grown size: the cotyledons and endosperm were undifferentiated, and the interior of the seeds was watery and gelatinous. Mature seeds were those obtained from fully grown, unopened fruits: they had fully developed cotyledons, endosperm, and seed coats. The hard outer coat of mature seeds was removed before use, leaving mainly cotyledons and endosperm. The entire seed was used from young fruits.

Radioactive materials were obtained from the Radiochemical Centre, Amersham, England. The absorbents used for column chromatography were silver nitrate-silica gel (Adsorbosil-CABN, 25% silver nitrate, 140/200 mesh, from Applied Science Laboratories, State College, Pa.), suitable for the estimation of cyclopropene fatty acids as described by Johnson et al. (10) and 60-100 mesh Florisil (Floridin Company, Florida).

Methods

Incubation Procedure. Tissue slices approximately 0.5-mm thick were cut transversely across seeds or fruits, weighed, and incubated in stoppered conical flasks containing a center well. Potassium hydroxide solution (10% w/v) was placed in the well, and filter paper was added. The incubation solution was 0.05M phosphate buffer (pH 6.6, 2.5 ml per g of slices), containing chloramphenicol (40 ppm). Potential precursors of the ring-methylene carbon, labeled with ^{14}C, were added in amounts governed by the period of incubation and the degree of activity required in the lipid. The flasks were shaken gently at 25C, and for incubations exceeding 4 hr the flasks were flushed with oxygen at intervals. After incubation the filter paper was removed, and the potassium hydroxide solution was rinsed from the center well. The contents of each flask were transferred to a Servall-Omnimix blender cup with chloroform-methanol 2:1 (v/v), and excess nonradioactive precursor was added to dilute any ^{14}C labeled precursor remaining.

Lipid Analysis. The crude lipid was extracted from the incubation mixtures, or from seeds alone, by homogenizing with chloroform-methanol (2:1 v/v) (11). Neutral lipid was separated from polar lipid on acid-treated Florisil by the method of Carroll (12). Polar lipid was either totally removed with methanol or separated into five fractions by using Carroll's solvent sequence. Neutral lipid was separated into classes by using Florisil which contained 6% (w/v) water (13).

The polar lipid fractions were examined by the method of Dawson et al. (14); the water-soluble hydrolysis products were separated by two-dimensional paper chromatography. Some polar lipid fractions were separated by chromatography on silica gel, by using concave gradients of methanol in chloroform (15). The separations were followed by the ninhydrin test and analysis for phosphate after digestion with perchloric acid (16). Thin-layer chromatography (TLC) was carried out by established methods (17). Phospholipids on TLC plates were detected by molybdenum blue spray reagent (18). It was shown that the polar lipids comprised a complex mixture largely of phospholipids.

Methyl esters of the lipid fatty acids were obtained by interesterification of the lipid classes with potassium methoxide in methanol (19). Triglycerides were refluxed under nitro-

TABLE I

Major Cyclopropane and Cyclopropene Fatty Acids in Young and Mature Seeds

Plant species	*Sterculia foetida*			*Brachychiton acerifolium*			*Brachychiton populneum*			*Gossypium hirsutum*		
Seed maturity	Young		Mature	Young		Mature	Young		Mature	Young		Mature
Total lipid (% of seed wet weight)	3.4		25.0	0.6		15.2	1.4		6.0	0.6		22.0
Polar lipid (PL) (% of total lipid)	31		6	69		8	53		6	34		5
Triglycerides (TG) (% of total lipid)	53		77	15		81	31		80	43		82
Cyclopropane and cyclopropene fatty acids in TG or PL[a]	*TG*	*PL*	*TG*	*TG*	*PL*	*TG*	*TG*	*PL*	*TG*	*TG*	*PL*	*TG*
C_{18} Branched-chain (malvalic)	11.6	....	6.5	20.5	....	8.2	54.3	2.6	7.2	0.8	0.1	0.2
C_{18} Dihydromalvalic	....	....	....	1.7	1.6	....	0.5	0.4	....	0.1	1.5	....
C_{19} Branched-chain (sterculic)	64.8	2.2	51.2	9.1	1.3	2.0	21.8	3.6	2.4	1.4	0.6	0.9
C_{19} Dihydrosterculic	4.6	4.7	0.4	3.5	0.9	1.4	0.8	1.2	....	1.2	0.7	0.1

[a] Estimated as methyl esters by Method B. Thus C_{18} branched-chain and C_{19} branched-chain give an estimate of the malvalic acid and sterculic acid respectively.

gen for 5 min and polar lipid fractions for 15 min. The extracted methyl esters were chromatographed on Florisil prior to analysis.

Estimation of Cyclopropane and Cyclopropene Fatty Acids. The fatty acid composition of the various lipid classes was deduced by treating the methyl esters in one of the following ways.

Method A: Hydrogenation. The methyl esters were hydrogenated in methanol with platinum oxide (10% of ester weight) for 30 min at room temperature and atmospheric pressure and were analyzed by gas-liquid chromatography (GLC).

Method B: Silver Nitrate-Silica Gel Chromatography. The foregoing hydrogenation method only gives a combined estimate of the cyclopropane-cyclopropene esters of each carbon number present in a mixture, and during this investigation a method was developed for the analysis of individual cyclopropane and cyclopropene fatty acids (10). This method is based on the observation that chromatography on silver nitrate-silica gel causes cyclopropene methyl esters to undergo ring opening, and all the products may be hydrogenated to yield branched-chain methyl esters. For example, methyl sterculate yields a mixture of methyl 9- and 10-methyloctadecanoates (C_{19} branched) in approximately a 1:1 ratio. This mixture gives a single peak on GLC, thus providing a direct estimate of the sterculate originally present. Likewise the GLC peak attributable to the C_{18} branched-chain esters provides a measure of the original malvalate.

Each methyl ester mixture was separated into saturated and unsaturated components by chromatography on silver nitrate-silica gel (Method 2 of Reference 10). The saturated methyl esters were analyzed directly by GLC, and the unsaturated esters were first hydrogenated, then analyzed. The compositions and weights of the two fractions were used to calculate the composition of the original methyl ester mixture.

Gas-Liquid Chromatography. A Packard model 7508 gas chromatograph with dual argon ionization detectors and dual 180 cm × 3 mm ID coiled glass columns was used. For analysis of nonradioactive methyl ester mixtures the columns were packed with Chromosorb G, coated with 1% Apiezon M and maintained at 185C. Radioactive mixtures were analyzed on columns of 10% Apiezon M on Chromosorb G at 220C. Peak areas were determined by ball and disc integrators (Disc Instruments Inc., California) coupled to the recorders.

Estimation of Radioactivity. The ^{14}C activity was determined in a Packard Tri-Carb liquid scintillation spectrometer, Model 3324. The scintillation fluid used for lipid samples was 2,5-diphenyloxazole (0.4% w/v) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (0.01% w/v) in toluene. Aqueous samples were counted in Bray's solution (20), and the $^{14}CO_2$ absorbed on the filter papers was counted by the method of Buhler (21), using the Diotol solution. Radioactive fatty acid methyl esters were separated by GLC at 220C. A 50:1 exit stream splitter was used, and the major split was trapped in anthracene cartridges in an automatic fraction collector (Packard Instrument Company). The ^{14}C content of each cartridge was then obtained directly by scintillation counting. The minor split was passed into the GLC detector, and the composition of the mixture was determined in the usual way. The specific activity of each methyl ester was calculated from the specific activity of the esters prior to GLC, the percentage composition of the mixture, and the percentage of the total

TABLE II

Incorporation of ^{14}C into the Fatty Acids of Fruit Slices of *Malva parviflora, Malva nicaensis,* and *Malvaviscus arboreus* after Incubation with ^{14}C-Labeled Compounds

Species	^{14}C Content of Hydrogenated Methyl Esters (Method A) as a Percentage of the Total ^{14}C Recovered after GLC							
	Malva parviflora[a]						*Malva nicaensis*[b]	*Malvaviscus arboreus*[b]
^{14}C Precursor	L-methionine [^{14}C-methyl]	^{14}C-Formate	^{14}C-Formaldehyde	3-^{14}C-L-Serine	2 ^{14}C-Malonate	1-^{14}C-Acetate	L-methionine [^{14}C-methyl]	L-methionine [^{14}C-methyl]
Fatty acid								
10-12	1.5	.....	3.3	0.7	5.7	0.3	.....	0.4
14	0.3	0.7	6.6	2.2	4.7	5.2	.....	.....
15	.....	.....	.....	.....	1.8	0.1	0.2	.....
16	1.3	12.6	32.5	53.2	38.7	49.3	0.2	.....
17 Cyclopropane	5.4	2.2	3.3	3.8	.....	0.8	1.8	6.4
17	2.1	0.4	.....	1.6	2.8	0.9	0.1	1.0
18 Branched-chain[c]	0.5	.....	1.4	.....	0.1	0.1	0.2	.....
18 Dihydromalvalic	1.3	1.1	1.9	0.4	1.2	0.2	2.1	0.5
18	2.6	12.3	30.0	26.1	40.5	42.0	1.9	1.0
19 Branched-chain[c]	14.1	4.0	2.5	1.1	1.6	0.3	2.1	1.9
19 Dihydrosterculic	66.3	65.1	15.8	10.7	1.6	0.6	88.2	87.5
19	4.9	1.9	2.5	0.8	1.3	0.2	3.2	1.7
^{14}C Content of (CPA+CPE)[d] as a percentage of total incorporation in fatty acids	92	74	27	17	6	2	98	98
μc ^{14}C Added per g of seed tissue	0.59	0.62	0.34	0.60	0.54	0.57	1.18	2.84
^{14}C Incorporated into fatty acids as a percentage of added ^{14}C	0.27	0.09	0.30	0.53	1.26	10.0	0.72	1.20
Specific activity of fatty acids (dpmpmg)[e]	480	150	340	700	7,720	41,970	2,690	1,860

[a] After 9 hr of incubation.

[b] After 22 hr of incubation.

[c] Branched-chain indicates a mixture of the branched-chain isomers which were derived from the corresponding cyclopropene fatty acid by hydrogenation (Method A).

[d] CPA+CPE was obtained from the sum of the radioactivity in C_{17} cyclopropane, C_{18} branched-chain, dihydromalvalic, C_{19} branched-chain, dihydrosterculic, and C_{19} esters. Contribution of C_{17} and C_{18} by-products of cyclopropene hydrogenation is not included in total.

[e] Disintegrations per minute per milligram.

counts associated with each fraction recovered from GLC.

Determination of Labeling Patterns of Methyl Sterculate –^{14}C. After incubation of L-methionine [^{14}C-methyl] with slices of young *Sterculia foetida* seeds, labeled methyl esters were fractionated by reversed-phase, liquid-liquid chromatography, and the methyl sterculate obtained was oxidized by techniques described (22). The ^{14}C contents of the various oxidation products and of the iodoform derived from the ring-methylene carbon were determined.

RESULTS

The effect of seed maturity on the content of the major cyclopropane and cyclopropene fatty acids on the triglycerides and polar lipids is shown in Table I.

In the young seed there was a high concentration of polar lipid, which had a low content of sterculic, malvalic, and dihydromalvalic acids and a high dihydrosterculic:sterculic ratio. Excepting those in cottonseed, the triglycerides of the young seeds contained a high proportion of sterculic and malvalic acids, also a low concentration of dihydromalvalic acid, and had a low dihydrosterculic:sterculic ratio. As the seeds developed, the total lipid content increased, but the proportion of triglycerides accumulated more rapidly. The C_{16} and C_{18} fatty acids of the triglycerides also increased, with a consequent dilution of the cyclopropene acids.

Table II shows the incorporation of ^{14}C into the fatty acids of fruit slices of *Malva parviflora, Malva nicaensis,* and *Malvaviscus arboreus* after incubation with various ^{14}C-labeled compounds. The specificity of incorporation of ^{14}C into the cyclopropane and cyclopropene fatty acids decreased in the order of methionine, formate, formaldehyde, serine, malonate, acetate. Under the conditions of incubation the

TABLE III

Incorporation of ^{14}C of L-methionine [^{14}C-methyl] into the Seed Lipids of Young Seeds of Various Plants of the Order Malvales

Species	Percentage of Lipid ^{14}C Recovered in Each Lipid Class					
	Brachychiton acerifolium	*Brachychiton populneum*	*Sterculia foetida*		*Gossypium hirsutum*	
Time of incubation (hr)	22	22	4	24	4	22
Hydrocarbons	0.1	.....	.....	.....	.....	.....
Sterol esters	1.6	1.1	0.4	0.4	1.4	3.0
Triglycerides	23.2	28.2	14.0	31.1	18.0	32.5
Sterols	20.2	22.9	6.0	14.8	6.3	29.8
Diglycerides	5.5	2.2	7.3	6.5	4.1	4.4
Monoglycerides	2.7	1.3	1.2	1.5	1.5	1.0
Free fatty acids	5.8	3.7	3.7	3.4	0.2	0.8
Phospholipid fatty acids	15.2	8.3	60.1	33.5	6.6	7.9
Phospholipid bases	25.7	32.5	7.3	8.8	59.2	20.5
Administered ^{14}C (μc per g of seed tissue)	5.0	8.0	4.0	4.0	5.5	5.5
Percentage ^{14}C recovered in lipid	6.9	5.2	4.1	5.3	0.6	1.0

fruit slices were actively synthesizing lipid, as shown by the high incorporation of 1-^{14}C-acetate into the straight-chain fatty acids.

After incubation with methionine, analysis of the hydrogenated methyl esters showed that dihydrosterculic acid contained the highest percentage of the label. However the relative amounts of the ^{14}C contributed by the natural dihydrosterculic acid and that derived from sterculic acid by hydrogenation cannot be determined by this method. Sterculic acid itself must have carried some label because both C_{19} normal and the C_{19} branched-chain isomers derived from sterculic acid were labeled. Hydrogenation converts malvalic acid, the main cyclopropene fatty acid of *Malva* species, largely to dihydromalvalic acid, but this contained only 1-2% of the label. In the three species examined, a significant proportion of the label was associated with a trace quantity of a methyl ester which had a retention time consistent with that of a C_{17} cyclopropane methyl ester.

The seeds of *Malva* plants are small and difficult to separate from the green fruit, their cyclopropene fatty acid content is low (CPA + CPE content of *Malvaviscus arboreus* is 0.5%, of *Malva parviflora* 3.0%, and of *Malva nicaensis* 3.3%), and they are generally unsuitable for investigations of this type. Seeds from trees of the *Sterculiaceae* were used in most of the later experiments, in which the biosynthesis of cyclopropane and cyclopropene fatty acids from the most specific precursor, methionine, was studied.

Table III shows the distribution of the ^{14}C of L-methionine [^{14}C-methyl] in the lipid classes of young *Brachychiton acerifolium* and *B. populneum* seeds after 22 hr of incubation, also

TABLE IV

Effect of Incubation Time on Incorporation of ^{14}C of L-methionine [^{14}C-methyl] into the Cyclopropane and Cyclopropene Fatty Acids[a] of the Phospholipids and Triglycerides of Young Seeds of *Sterculia foetida* and *Gossypium hirsutum*

	Cyclopropane and cyclopropene fatty acids[b]	*Sterculia foetida*				*Gossypium hirsutum*			
		Phospholipids		Triglycerides		Phospholipids		Triglycerides	
		4 hr	24 hr	4 hr	24 hr	4 hr	22 hr	4 hr	22 hr
Percentage recovered activity after GLC[c]	C_{17} Branched-chain (17 cyclopropene)	—	0.1	—	0.1	—	0.04	—	0.3
	C_{17} Cyclopropane	0.1	0.2	—	0.2	—	0.2	—	0.3
	C_{18} Branched-chain (malvalic)	0.02	0.04	2.7	1.7	—	0.9	—	4.9
	C_{18} Dihydromalvalic	0.2	0.25	0.4	0.4	—	2.9	5.6	6.1
	C_{19} Branched-chain (sterculic)	4.0	13.4	44.2	44.7	4.4	16.0	23.7	25.8
	C_{19} Dihydrosterculic	94.7	84.5	51.3	50.8	93.0	77.6	63.9	46.7
Specific activity[c] (dpmpmg x 10^{-3})	C_{18} Branched-chain (malvalic)	—	—	12	21	—	—	—	10
	C_{18} Dihydromalvalic	—	—	—	—	—	32	34	104
	C_{19} Branched-chain (sterculic)	258	327	36	100	54	437	10	31
	C_{19} Dihydrosterculic	1,682	1,589	581	1,608	983	1,818	33	66
	Total methyl esters	120	82	6	17	7	16	1	2

[a] For percentage weight composition, see Table I.

[b] See footnote, Table I.

[c] Dash indicates insufficient activity or mass for accurate estimation.

TABLE V

Incorporation of ^{14}C of L-methionine [^{14}C-methyl] after 22 Hr of Incubation into the Cyclopropane and Cyclopropene Fatty Acids[a] of Phospholipids and Triglycerides of Young Seeds of *Brachychiton acerifolium* and *B. populneum*

		B. acerifolium			*B. populneum*		
Cyclopropane and cyclopropene fatty acids[b]		Phospholipid 1[d]	Phospholipid 5[d]	Triglycerides	Phospholipid 1[d]	Phospholipid 5[d]	Triglycerides
Percentage recovered activity after GLC[c]	C_{17} Branched-chain (17 cyclopropene)	0.08	0.2	0.02	0.1	0.2	0.1
	C_{17} Cyclopropane	0.5	0.4	0.3	3.2	3.7	2.6
	C_{18} Branched-chain (malvalic)	0.06	0.1	7.2	0.1	0.2	.07
	C_{18} Dihydromalvalic	2.0	0.9	1.3	7.1	7.0	9.6
	C_{19} Branched-chain (sterculic)	9.7	30.4	38.4	16.0	20.4	51.1
	C_{19} Dihydrosterculic	86.5	67.1	50.3	65.7	56.0	27.5
Specific activity[c] (dpmpmg x 10^{-3})	C_{17} Branched-chain (17 cyclopropene)	—	—	—	—	—	193
	C_{17} Cyclopropane	—	—	—	366	776	1,282
	C_{18} Branched-chain (malvalic)	—	—	42	1	3	0.3
	C_{18} Dihydromalvalic	42	—	90	449	816	371
	C_{19} Branched-chain (sterculic)	222	2,366	499	114	221	46
	C_{19} Dihydrosterculic	727	2,402	1,699	1,143	2,448	654
	Total methyl esters	34	170	118	30	31	20

[a] For percentage weight composition, see Table I.

[b] See footnote, Table I.

[c] Dash indicates insufficient activity or mass for accurate estimation.

[d] Phospholipids 1 and 5, eluted with 5% v/v methanol-chloroform and 100% methanol respectively.

the effect of incubation period on this distribution in seeds of young *Sterculia foetida* and *Gossypium hirsutum*. In all these plants, irrespective of the period of incubation, the main sites of ^{14}C incorporation were the triglycerides, the sterols, the phospholipid fatty acids, and the phospholipid base moieties. The label associated with sterols and triglycerides increased with the time of incubation. The total incorporation of label into the triglycerides and phospholipid fatty acids does not give a true picture of the incorporation of the ^{14}C of the methyl group of methionine into cyclopropane and cyclopropene fatty acids for the cyclopropene fatty acid content of the triglycerides is high whereas that of the phospholipids is low (Table I).

The effect of the incubation period on the incorporation of ^{14}C of L-methionine [^{14}C-methyl] into the phospholipid and triglyceride fatty acids of the young seeds of *Sterculia foetida* and *Gossypium hirsutum* is shown in Table IV. Only the cyclopropane and cyclopropene fatty acids contained significant amounts of radioactivity. The dihydrosterculic acid of the phospholipids was, in both seeds, the initial product for it carried at least 93% of the label after 4 hr of incubation. This percentage of label decreased after 24 hr of incubation, and the ^{14}C content and specific activity of the phospholipid-sterculate (C_{19} branched-chain isomers, Table IV) increased, suggesting that the dihydrosterculic acid was desaturated to sterculic acid. In many instances it was not possible to calculate specific activities; the content of some labeled fatty acids was too low to produce a response from the mass detector. However, with the phospholipids, the percentage of recovered counts associated with dihydromalvalate was higher than that of the C_{18} branched-chain fatty acids, suggesting again that the cyclopropane fatty acid is desaturated to the cyclopropene.

The relative amounts of label incorporated into the fatty acids of the phospholipids and triglycerides of *Brachychiton acerifolium* and *B. populneum* seeds after 22 hr of incubation are shown in Table V. In both species the phospholipids were separated into five fractions, but only the results for the phospholipid 1 (phosphatidic acid, phosphatidyl ethanolamine, and phosphatidyl glycerol) and phospholipid 5 (phosphatidyl choline) are recorded in the table because the incorporation of radioactivity into the intermediate fractions was essentially the same as in phospholipid 1. The ^{14}C activity of the phospholipid cyclopropane esters was always higher than that of the corresponding cyclopropene esters, confirming that the cyclopropene fatty acid is derived from the cyclopropane, as in the other seeds examined.

Some radioactivity was found before C_{17} and beyond C_{19} in all species examined. This activity was found to be located at retention values corresponding to lower and higher members of an homologous series of cyclopropane and cyclopropene fatty acids.

The distribution of the ^{14}C between the in-

dividual fatty acids of the triglycerides and phospholipids of mature seeds was determined, but results are not given because the deductions were the same as those obtained from young seeds.

Methyl dihydrosterculate (specific activity 200,000 dpmpmg) and a fraction containing largely methyl sterculate (15,000 dpmpmg) were obtained (22) from the labeled methyl esters of young *Sterculia foetida* seeds. Radioactive methyl n-octyl ketone (30,000 dpmpmg) was identified in the neutral products of the periodate-permanganate oxidation of the labeled sterculate (calculated specific activity 29,600 dpmpmg). Iodoform prepared from the labeled ketone retained all the activity, showing that the ^{14}C was located on the α-methyl group of the ketone, which was originally derived from the methylene bridge carbon atom of the sterculate. This showed that the radioactive methyl sterculate was specifically labeled on the methylene carbon atom.

Further proof of this finding was provided by an examination of the acidic products of the oxidation-hydrolysis of the labeled sterculate. The steam-volatile acids, mainly pelargonic acid, showed little activity. The acids not volatile in steam contained inactive azelaic acid and radioactive 9-keto-decanoic acid as the main components. Of the three acids only the radioactive keto-acid retained the methylene carbon atom of the parent sterculate, thus again showing that the methylene carbon atom was specifically labeled.

DISCUSSION

The ^{14}C of L-methionine [^{14}C-methyl] is incorporated into the dihydrosterculate and sterculate residues in the phospholipids and triglycerides. The methyl group is probably transmitted as S-adenosyl methionine, as has been demonstrated in bacteria (2,3,4), and the derived methylene group then adds across the double bond of a fatty acid. To form dihydrosterculic acid (9, 10-methylene octadecanoic acid) the fatty acid precursor must be oleic acid (octadec-9-enoic acid). Biosynthesis of cyclopropane fatty acids in these seeds is therefore analogous to that in bacterial systems, where it has been shown (2,4) that *cis*-vaccenic acid on phosphatidyl ethanolamine is the precursor of lactobacillic acid. In the seeds studied the complex composition of the polar lipids precluded the isolation of pure single phospholipids, and it was not possible to determine whether there was a specific phospholipid essential for the synthesis of dihydrosterculic acid.

The percentage of label incorporated into the initial product, the dihydrosterculate on the phospholipid, decreased during the time of incubation whereas that of the sterculate on the phospholipid rose, suggesting that dihydrosterculic acid is desaturated to sterculic acid. This conclusion does not support the postulated biosynthesis of sterculic acid by the addition of a methylene group to the acetylenic bond of stearolic acid (7).

While the biosynthetic pathway for dihydrosterculic and sterculic acids has been deduced from the results presented herein, the route of synthesis of the other members of the homologous series is not immediately apparent. The route of synthesis of malvalic acid is of interest because it forms the major cyclopropenoid component of some of the seeds examined and because the ring is in the 8,9-position in the carbon chain. It is likely, from the observed activities of dihydromalvalic and malvalic acids, that dihydromalvalic acid is the immediate precursor of malvalic acid; but it is not apparent whether the dihydromalvalic acid arose by α-oxidation (23) of dihydrosterculic acid or from the addition of a methylene group to a C_{17} mono-unsaturated fatty acid. The latter compound is present in small amounts in these seeds, but the position of the double bond is unknown. The work of Smith and Bu'Lock (7) does not exclude the possibility that the C_{17} mono-unsaturated fatty acid could be formed from α-oxidation of oleate.

The labeled C_{17} cyclopropane fatty acid which was observed could have been formed from either palmitoleic acid or from dihydrosterculic acid by chain shortening by β-oxidation. Wood and Reiser (24) have shown that *cis*- and *trans*-3,4-methylene dodecanoates accumulate in the tissues of rats fed *cis*- and *trans*- dihydrosterculate, and they conclude that these arose by β-oxidation of the dihydrosterculic acids. In the seeds, α-oxidation (23) or β-oxidation of dihydrosterculic and sterculic acids could lead to the trace amounts of lower cyclopropane fatty acids, and higher homologs could be formed by the addition of C_2 units. Alternatively, although the enzyme responsible for the formation of dihydrosterculic acid is most active for the addition of the methylene group to oleic acid, it could to a lesser extent add the methylene group to other monounsaturated fatty acids. The results do not suggest which of these pathways predominates.

For all three types of seeds examined, namely, those rich in sterculic acid, those rich in malvalic acid, and those with a low cyclo-

propene fatty acid content, the results have demonstrated the same biosynthetic pathway for the formation of the cyclopropene fatty acids.

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The Reaction of Methyl Sterculate and Malvalate with Silver Nitrate-Silica Gel and Its Use as a Basis for the Estimation of Cyclopropene Fatty Acids

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ABSTRACT

When in contact with silver nitrate-silica gel, methyl esters of cyclopropene fatty acids undergo ring opening to yield pairs of isomers with methylene, hydroxymethyl, or nitratomethyl side-chains at the original ring positions. Thus the main products from methyl sterculate were the methyl 9(or 10)-methylene octadec-10(or 8)-enoates, and, in lesser quantities, the methyl 9(or 10)-(nitratomethyl)-octadec-9-enoates and the methyl 9(or 10)-(hydroxymethyl)-octadec-9-enoates. Hydrogenation quantitatively converted this mixture of isomeric pairs to a mixture of methyl 9- and methyl 10-methyloctadecanoates.

Chromatography on silver nitrate-silica gel, followed by gas chromatography of the hydrogenated products, has been used as the basis of a new method for estimating individual cyclopropene and cyclopropane acids in lipids.

INTRODUCTION

CHROMATOGRAPHY on silver nitrate-silica gel is extensively used for the separation of methyl esters of fatty acids of varying degrees of unsaturation (1,2) and accordingly appeared to provide a suitable means for separating cyclopropene methyl esters (3). However, Cornelius et al. (4) found the method to be unsuitable for this purpose. Kircher (5) later reported that sterculene in dilute solution in organic solvents reacts wth silver nitrate; the nature of the end-product and its rate of formation both are largely determined by the character of the solvent.

It was observed in this laboratory that methyl sterculate applied in hexane to thin-layer plates of silver nitrate-silica gel produced three major products. A closer study of these, and of the reaction products similarly derived from methyl malvalate, provided the basis for developing a suitable quantitative method for estimating individual cyclopropene and cyclopropane fatty acids in lipids. An account of this work is now given.

EXPERIMENTAL

Materials and Methods

Methyl sterculate was isolated from *Sterculia foetida* seed oil by the method of Kircher (6), and methyl malvalate was prepared from the same source (7).

Silver nitrate-silica gel (Adsorbosil-CABN, 25% silver nitrate 140/200 mesh from Applied Science Laboratories, Pennsylvania) was used for column chromatography. For thin-layer chromatography (TLC), Merck Kieselgel G was used with hexane:diethyl ether:acetic acid (90:10:1) as solvent; alternatively plates were used that had been freshly prepared from Kieselgel G impregnated with 25% by weight of silver nitrate (8); the solvent was hexane:diethyl ether (9:1). In either case, methyl esters were detected by spraying with 2′, 7′-dichlorofluorescein solution (0.2% w/v in ethanol). On normal Kieselgel G plates, spraying with an ethanolic solution of silver nitrate (1% w/v) produced a brown spot at the location of each cyclopropene compound. This reaction appears to be specific for the cyclopropene ring; the limit of detection for sterculate or malvalate was approximately 5 μg.

The gas chromatograph was a Packard Model 7508 with dual argon ionization detectors and dual 180 cm by 3 mm I.D. columns of coiled glass. Stationary phases were 5% diethylene glycol adipate and 1% Apiezon M (APM), both on Chromosorb G. Operation was isothermal at 185C. Later the organosilicon-polyester packings 16% ECNSS-S and 14% ECNSS-M on Gas-Chrom P (Applied Science Laboratories) were also used. The instruments used to record the spectra indicated were Perkin-Elmer 221 (infrared), Beckman DK2A (ultraviolet), Varian 60 (NMR), and Atlas CH4 (mass).

Unsaturated esters and the reaction products of methyl sterculate and malvalate were hydrogenated in methanol over platinum oxide (10% of ester weight) for 30 min at room tempera-

ture and atmospheric pressure. Unsaturated fractions were oxidized with periodate-permanganate (9,10), and the resultant mono- and dicarboxylic acids, after being methylated with boron trifluoride-methanol reagent, were identified and estimated by gas-liquid chromatography (GLC).

Isolation of Reaction Products

In general, methyl sterculate or methyl malvalate was allowed to react with the silver nitrate-silica gel by passing a solution of the ester in hexane through a column suitably packed with this material. The reaction products were separately recovered by elution with mixtures of hexane and successively increasing amounts of diethyl ether or benzene. Several such chromatograms were run on each ester. Details of a typical run on methyl malvalate follow.

"The methyl malvalate was first chromatographed on a Florisil column (11) to remove traces of oxidized impurity (7). This purified ester (300 mg), dissolved in hexane, was applied to silver nitrate-silica gel (35 g) packed in a glass column (15 mm I.D.) wrapped in black tape. Within a few seconds a brown stain appeared at the top of the column. The initial eluant was 5% (v/v) diethyl ether in hexane, but the proportion of ether was increased stepwise to 100% in successive eluants (Fig. 1). After removing the solvent from 25-ml fractions collected in tared tubes, the residues were weighed (total material recovered 301 mg)."

All fractions were examined by TLC on Kieselgel G and on Kieselgel G-silver nitrate (Fig. 1). For each ester, fractions which showed a single spot on both types of TLC plate and had also identical IR spectra were combined (shaded areas in Fig. 1) to give three main products, referred to hereafter as products A, B, and C. All experiments with methyl malvalate yielded closely similar quantities of these same products while those with methyl sterculate gave analogous products, also in reproducible yields.

Products A, B, and C from methyl sterculate and from methyl malvalate were examined before and after hydrogenation by GLC on two phases (APM and 14% ECNSS-M) by TLC on the two systems mentioned above; and the UV, IR, NMR, and mass spectra were recorded. Product B from methyl sterculate was also submitted to oxidative fission.

Procedures for Estimating Acids

The following two methods have, as their basis, findings reported and discussed later in this paper.

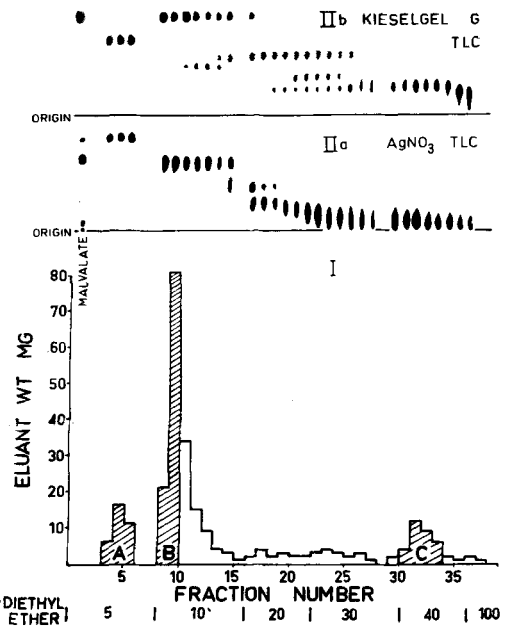


FIG. 1. The reaction of methyl malvalate with silver nitrate-silica gel.

I Reaction products eluted from the silver nitrate-silica gel column by increasing concentrations of diethyl ether in hexane. Shaded areas indicate pooled fractions designated products A, B, and C.

II Diagrammatic representation of TLC of approximately 250 μg of individual fractions and of methyl malvalate. Sprayed with 2', 7'-dichlorofluorescein.

a) TLC plates, Kieselgel G impregnated with $AgNO_3$ (25%); solvent 9:1 hexane: diethyl ether. R_f value of the most mobile spots, 0.49.

b) TLC plates, Kieselgel G; solvent, 90:10:1 hexane:diethyl ether:acetic acid. R_f of the most mobile spots, 0.48.

Method 1. Simplified Procedure. The fatty acid methyl ester mixture (approximately 30 mg) is applied in hexane to silver nitrate-silica gel (3 g) packed in a 15-mm tube. The added material is then completely stripped from the column with diethyl ether (100 ml). The recovered eluate containing reaction products which originate from the cyclopropene methyl ester, the normal saturated and unsaturated methyl esters, and the cyclopropane esters is then hydrogenated and analyzed by GLC. On APM columns there is good resolution of the unchanged cyclopropane esters, the normal saturated esters, and the methyl branched-chain esters representing the original cyclopropene esters.

Method 2. Procedure Involving Separation of Saturated and Unsaturated Esters. The methyl ester mixture (not more than 30 mg) is applied to a 10-g column, as in Method 1. The saturated esters, including cyclopropane esters, are then completely eluted with 30% v/v benzene-hexane; the volume required is dependent on the composition and amount of the mixture of esters fed into the column. For 20-30 mg samples of seed lipids, this volume, determined by fraction analysis, was found to be 160 ml. Unsaturated esters together with cyclopropene ester reaction products are next eluted from the column with diethyl ether (100 ml) and, after the solvents are removed, the mixed product is hydrogenated. This hydrogenated fraction and the original saturated ester fraction are each analyzed by GLC on APM. From the compositions and weights of these two fractions the composition of the orginal ester mixture is finally calculated.

Evaluation of the Methods

To establish the accuracy of Methods 1 and 2, methyl esters from the seed lipid of immature *Brachychiton populneum* seeds, rich in malvalic acid, and from the seed lipid of *Sterculia foetida*, rich in sterculic acid, were accurately diluted with various amounts of methyl esters of the fatty acids of wheat germ oil and analyzed by Method 1 and also by GLC of their hydrogenation products.

In the hydrogenation method, the quantity of methyl sterculate originally present was calculated from the total yield of the hydrogenation products, namely, methyl dihydrosterculate, methyl 9- and 10-methylstearates (C_{19} branched-chain isomers), and methyl nonadecanoate (C_{19}) (12). One of the hydrogenation products of methyl malvalate is methyl stearate, which cannot be distinguished from methyl stearate originally present or from that derived from the hydrogenation of octadecenoates. However it is possible to determine the total malvalate content by direct hydrogenation if an estimate can also be made of the C_{18} esters originally present. Such an estimate is provided by the silver nitrate method.

In the absence of methyl malvalate, the hydrogenation method and the silver-nitrate method give similar estimates of the saturated esters produced whereas, when methyl malvalate is present, the hydrogenation method gives a higher methyl stearate value than the silver-nitrate method. The difference is attributable to methyl stearate contributed by methyl malvalate. This increment, together with the amounts of methyl dihydromalvalate and of C_{18} branched-chain esters produced by the hydrogenation method, was used to obtain the total content of methyl malvalate.

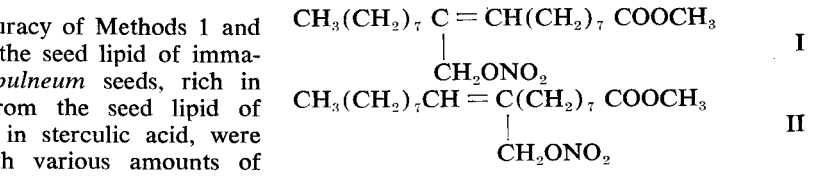
In another series of tests a known mixture by weight of methyl palmitate and methyl sterculate was analyzed by Method 1, by the hydrogenation-GLC method, and by the method of Raju and Reiser (13), in which the products of reaction between the cyclopropene ester and methyl mercaptan are estimated by GLC.

Finally, methyl esters from seed lipids containing cyclopropene acids were analyzed by Method 2, also by the direct hydrogenation-GLC method mentioned above.

RESULTS AND DISCUSSION

Structure of Products Recovered

Product A. The combined evidence, summarized below, established that product A from methyl sterculate was a mixture of two nitrated products having structures I and II respectively.



I

II

The IR spectrum provided conclusive evidence of the covalent nitrate group, bands at 1635, 1278, 862, 760, and 696 cm^{-1} agreeing in wave number and relative intensity with those quoted by Brown (14) for liquid mononitrates. The methyl ester group (1737 cm^{-1}) of the original material was present. The UV spectrum showed no absorption in the region 220-350 mμ. The integrated NMR spectrum was in agreement with the structures I and II. In particular, a "triplet" centered at δ 5.0 represented the two protons of the $-CH_2ONO_2$ sidechain. This value is in accord with the value of δ 4.48 for the same protons in amyl nitrate when due allowance is made for a further shift of approximately δ 0.5 for the double bond in the β-position (15).

The mass spectrum showed no molecular ion corresponding to m/e = 371 for structures I and II. But an apparent molecular ion at m/e = 324 was present and persisted at low energy (9eV) along with a fragment at m/e = 30. These observations strongly suggest that product A from methyl sterculate underwent thermal change in the mass spectrometer: elimination of HNO_2 (indicated as NO^+, with m/e = 30) would give a carbonyl product with a molecular weight of 324. Other peaks at m/e 292, 293, prominent in the spectra of products B

and C from the same ester, are attributed to loss of the side-chain.

The thermal instability of product A from methyl sterculate was confirmed by heating it in vacuo in a 6-mm glass tube at 180-190C; the pressure was reduced from 3 mm to 0.5 mm. Distillate was collected in a cool part of the tube, and gaseous products were condensed in a liquid air trap. The amount of this condensate corresponded to the decomposition of approximately one-fifth of the original material and gave, with the method of Kefford (16), a positive indication of NO_2. The distillate, examined by TLC and by its IR spectrum, contained unchanged material and several other products that are discussed further below.

Product A from methyl sterculate had, after hydrogenation, an IR spectrum almost identical with that of an authentic specimen of methyl 10-methylstearate. The mass spectrum however clearly showed that the hydrogenated product was a mixture of 9- and 10-methylstearates (17), and from the relative intensity of peaks at m/e 199 and 213 the 9-methyl ester appeared to be in slight excess.

Product B. The second product from methyl sterculate was established as a mixture of the dienes III and IV on the following evidence:

$$CH_3(CH_2)_7\ C(=CH_2) - CH = CH(CH_2)_6\ COOCH_3 \qquad III$$

$$CH_3(CH_2)_6\ CH = CH - C(=CH_2)(CH_2)_7\ COOCH_3 \qquad IV$$

The material showed a single peak during GLC on polar and nonpolar phases. Retention times related to methyl stearate were 1.488 (APM) and 1.945 (14% ECNSS-M) for product B from sterculate, and for the corresponding product from malvalate were 0.930 and 1.425 respectively.

The mass spectrum of product B from sterculate had a strong molecular ion peak and established the molecular weight as 308. The IR spectrum showed strong absorption bands attributable to $CH_2 = CR_1R_2$ (887 and 3085 cm^{-1}) and to conjugated diene (1610 and 1650 cm^{-1}) whereas the UV spectrum showed intense absorption at 230 mμ (ε_{max} approx 16000), indicating the presence of a disubstituted butadiene group. The NMR spectrum and the integrated intensities of the peaks were in general agreement with structures III and IV. In particular, there was clear evidence (δ 4.85) for the two protons of the methylene side-chain and for the two single protons adjacent to a conjugated double bond (δ 5.8) (15).

Like product A, product B from methyl sterculate gave, when hydrogenated, a mixture of methyl 9- and 10-methylstearates, but the mass spectrum showed that the proportion of 9-methyl ester in this mixture was higher than in that from product A. Oxidative fission of product B with permanganate-iodate confirmed the double bond positions in III and IV, and the molar ratio of the oxidation products, azelaic and suberic acids, showed that the ratio of IV:III was approximately 1.4:1.

Product C. The evidence indicated that product C from methyl sterculate was a mixture of the unsaturated hydroxylated compounds V and VI.

$$CH_3(CH_2)_7\ C(CH_2OH) = CH(CH_2)_7\ COOCH_3 \qquad V$$

$$CH_3(CH_2)_7\ CH = C(CH_2OH)(CH_2)_7\ COOCH_3 \qquad VI$$

In GLC the products from methyl sterculate and from methyl malvalate were retained at 185C by the 14% ECNSS-M column. On APM both products gave single peaks of long retention-times (sterculate, 3.228; malvalate, 2.080).

The mass spectrum gave a small molecular ion peak at m/e 326, identifiable as such by the presence of M-2, M-18, and M-31 peaks typical of a primary alcohol of high molecular weight (18). The IR spectrum showed the presence of hydroxyl around 3400 cm^{-1} and primary hydroxyl at 1010 cm^{-1}. There was also evidence of an isolated double bond, namely, a small band at 1640 cm^{-1}. There was no absorption in the UV. The NMR spectrum and the integration of peaks agreed with the above structures. There was particular evidence for the allylic alcohol group $-\overset{|}{C} = \overset{|}{C} - CH_2OH$ with 2 (or 3) protons at δ 4.1 (15), and for one vinyl proton (δ 5.5).

Like products A and B, product C also gave, when hydrogenated, a mixture of 9- and 10-methylstearates. In this instance the mass spectrum indicated that these end-products were in the same proportion as in the mixture derived from hydrogenation of product A.

Products A, B, and C from pure methyl malvalate were examined likewise and, on similar evidence, were found to comprise corresponding mixtures of analogs of I to VI, i.e., compounds with one carbon atom less and with the branches at the 8 C or 9 C atoms.

Several minor products formed by the reaction of methyl sterculate or malvalate on silver

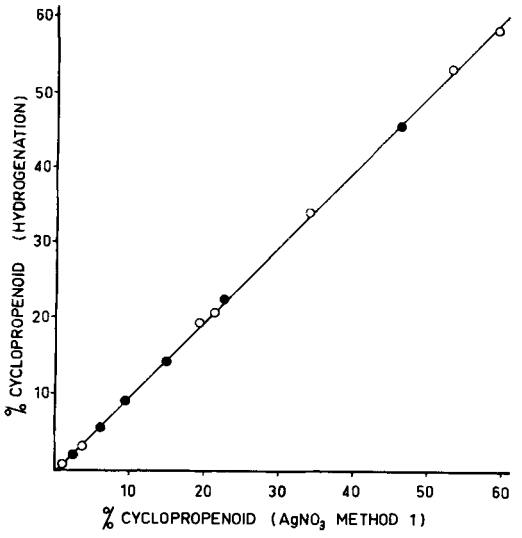


FIG. 2. Correlation of malvalate and sterculate contents of seed lipids as estimated by the direct hydrogenation method and by the silver-nitrate technique, Method 1.
● = methyl sterculate; o = methyl malvalate.

nitrate-silica gel are apparent in Fig. 1 as thin-layer spots lying between those of products B and C. However, because the mixture of 9- and 10-methyl branched-chain esters accounts for approximately 97% of the hydrogenated products of the reaction, it is clear that, on hydrogenation, some of these minor products must give end-products similar to those given by the main products A, B, and C.

One minor product, which on hydrogenation did not form methyl branched-chain esters, was isolated in small amount after chromatography of methyl sterculate on silver nitrate-silica gel. The IR and mass spectra of this product, which was eluted from Florisil by 15% diethyl ether in hexane during re-chromatography, indicated it to be a conjugated ketone of molecular weight 324. Kircher (5) showed that, in nonhydroxylic solvents, the major product of the reaction between silver nitrate and sterculene is a conjugated ketone. From the reaction between silver nitrate and methyl sterculate in acetonitrile, under conditions similar to those of Kircher, a conjugated ketone ester was isolated with IR and mass spectra identical with the above minor product. Presumably, like products A, B, and C, the conjugated ketone ester is a mixture of isomers, in this case, of methyl 10-methylene-9-oxostearate and methyl 9-methylene-10-oxostearate.[1]

Four classes of compounds have thus been positively identified among the reaction products of methyl sterculate or methyl malvalate and silver nitrate-silica gel, namely, a nitrate ester (product A), a diene (product B) an alcohol (product C), and a conjugated ketone. There is strong evidence that the nitrate ester is the primary product and gives rise to the other three by elimination or hydrolytic reactions. First, alkyl nitrates are already known to undergo hydrolytic decomposition to yield simultaneously an alcohol, an olefin, and a carbonyl compound (19). Also TLC and IR spectra indicated that products B and C and the conjugated ketone were present in the distillate from product A; some decomposition occurred during its distillation, with elimination of HNO_2. Other evidence is that peaks corresponding to products B and C and the conjugated ketone were evident in the gas chromatogram of product A.

In the mass spectrometer, conditions are apparently such that elimination of HNO_2 proceeds smoothly to give almost exclusively the carbonyl product. Except for the intensity of the m/e 30 ion peak and the presence of a small m/e 308 peak, attributed to the diene ester, the spectra of product A and the conjugated ketone were almost identical.

Estimation of Fatty Acids

Fig. 2 shows that, for seed lipids containing no detectable cyclopropane acids, malvalate and sterculate contents determined by Method 1 of the silver-nitrate technique are strongly and linearly correlated with those given by the direct hydrogenation-GLC method. For cyclopropene fatty acid contents below 1%, neither method is likely to have given accurate results because the GLC technique is not accurate for small peaks incompletely resolved from large peaks. Although above 5% cyclopropene content both methods gave almost identical figures, at lower contents of cyclopropene fatty acids the hydrogenation method generally gave lower values than the silver-nitrate method. In this region the direct-hydrogenation, GLC technique is less accurate because the cyclopropene content must be estimated from the sum of the GLC peaks representing respectively the derived cyclopropane, the straight-chain esters, and the branched-chain esters; in the silver-

[1]The results reported in this paper and elsewhere (20) were obtained with batches of Adsorbosil CABN 1420 purchased in 1964 and 1966. In 1965 several batches (e.g., No. 9-15A) gave anomalous results in that appreciable amounts of the conjugated ketone esters were formed. The reason for the difference between these batches is being sought, but meanwhile the manufacturers have indicated that they are able to supply Adsorbosil with the desired characteristics.

TABLE I

Comparison of GLC Results Obtained by Silver Nitrate Technique (Method 2) and After Direct Hydrogenation

	Fatty acid methyl ester composition (wt%)								
	Sterculia foetida seed lipid			*Brachychiton acerifolium* seed lipid			*Brachychiton populneum* seed lipid triglycerides		
Fatty Acid	Hydrogenation	$AgNO_3$		Hydrogenation	$AgNO_3$		Hydrogenation	$AgNO_3$	
		Saturated	Unsaturated		Saturated	Unsaturated		Saturated	Unsaturated
C_{14}	0.1	0.2	—	0.3	0.2	—	—	—	—
C_{14}:1	—	—	—	—	—	0.1	—	—	—
C_{15}	0.1	0.1	—	0.2	0.2	—	—	—	—
C_{16}	23.5	22.9	—	18.0	16.8	—	18.3	17.5	—
C_{16}:1	—	—	0.4	—	—	0.9	—	—	0.7
C_{17}	0.3	0.1	—	2.7	—	—	1.1	—	—
C_{17}:1	—	—	0.2	—	—	3.0	—	—	1.6
C_{18} branched-chain (malvalic)	0.4	—	6.5	0.6	—	8.2	1.0	—	7.2
Dihydromalvalic	5.3	—	—	6.6	—	—	5.7	—	—
C_{18}	18.7	1.4	—	68.4	1.2	—	71.5	3.6	—
C_{18} Unsaturates	—	—	16.5	—	—	66.0	—	—	67.0
C_{19} branched-chain (sterculic)	3.8	—	51.2	0.1	—	2.0	0.1	—	2.4
Dihydrosterculic	37.2	0.4	—	2.6	1.4	—	2.2	—	—
C_{19}	10.6	—	—	0.1	—	—	0.1	—	—

nitrate method the cyclopropene value is represented only by the peak corresponding to the branched-chain esters.

A mixture of methyl palmitate and methyl sterculate containing 78.5% by weight of the latter was analyzed for sterculate content by three methods, each in quadruplicate, with the following mean results: direct hydrogenation method, 78.1%; silver nitrate, Method 1, 76.1%; methyl mercaptan method (13), 76.8%. In the silver-nitrate method approximately 1% of the methyl sterculate was converted to the unsaturated ketone esters previously mentioned. Reaction of the sterculate-palmitate mixture with methyl mercaptan (13) also yielded approximately 1% of unidentified products, which could have been derived only from sterculate since they were not formed when methyl palmitate, stearate, oleate, or linoleate were treated with methyl mercaptan under the same conditions.

The GLC data pertaining to seed lipids containing cyclopropene acids and analyzed by the silver-nitrate technique, Method 2, and also after direct hydrogenation, are compared in Table I. Agreement between the two methods is excellent, provided allowance is made for certain shortcomings of the direct hydrogenation method. These are that the cyclopropene acid content must be derived from the sum of its three ester products and, particularly, that original cyclopropane and derived cyclopropane acids cannot be differentiated; nor can a value for stearate derived from malvalate be obtained directly because of stearates originally present or derived from unsaturated C_{18} straight-chain esters.

Both of the silver-nitrate methods described are thus distinctly superior to the direct hydrogenation method. When values for the individual cyclopropene and cyclopropane acids are the principal requirement, the simpler Method 1 is preferred. A more complete analysis of a seed lipid is possible by Method 2, which permits complete and separate estimation of the unsaturated compounds. Also, when the content of cyclopropane acids is low, concentration and better resolution by GLC is facilitated by the prior removal of the unsaturates. For this last reason, Method 2 was widely used in recent biosynthetic studies in this laboratory (20).

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The Phospholipid Composition of Plasma in Various Mammalian Species

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ABSTRACT

Plasma phospholipids in several common mammalian species, including cat, cow, dog, goat, guinea pig, horse, pig, rabbit, rat, and sheep, were analyzed by using chromatographic and spectrophotometric methods. Lipids were extracted from plasma with chloroform-methanol 2:1 (v/v) and freed of nonlipid material by passage through a Sephadex column. The phospholipids were separated by two-dimensional thin-layer chromatography (TLC). Spots were identified by spray reagents, also by infrared spectrophotometry. The relative distribution of the phospholipids was determined by phosphorus analysis on the spot scraped off the TLC plate.

Lecithin, lysolecithin, and sphingomyelin were found in the plasma of all species and accounted for more than 95% of the phospholipids except in the rodents. Lecithin was without exception the major phospholipid in plasma (56 to 83%). Lysolecithin and sphingomyelin content varied between 8 and 23% and 6 and 15% respectively. Phosphatidyl ethanolamine and phosphatidyl inositol were the only noncholine-containing phospholipids detected (detection limits 0.2%) in the plasma of these species. Together these compounds usually made up less than 5% of the total phospholipid. Rodents were an exception, especially the guinea pig, which had 21.7% phosphatidyl ethanolamine.

INTRODUCTION

THE PHOSPHOLIPIDS of human plasma[1] have been studied extensively in recent years (1-5), but detailed analyses of the plasma phospholipid in other species, except for the rat (6-9), are limited or unavailable. Dawson et al. (10) present data for cow, goat, horse, pig, and sheep which were obtained with hydrolytic techniques coupled with paper chromatography of the hydrolysis products. Earlier Taurog et al. (11), Entenman and Chaikoff (12), Sinclair (13), and Axelrod et al. (14) had reported values for the plasma phospholipids in more limited series by using a variety of analytical techniques, but differences in methods and manner of reporting the data have made it difficult to compare values between species.

This report presents the results of an investigation of the phospholipid distribution in the plasma of 10 common mammalian species: cat, cow, dog, goat, guinea pig, horse, pig, rabbit, rat, and sheep. The analyses were performed by two-dimensional thin-layer chromatography (TLC), combined with spectrophotometric determination of phosphorus and with infrared spectrophotometry.

EXPERIMENTAL PROCEDURE

Materials

Standard phospholipid preparations were obtained from General Biochemicals, Chagrin Falls, O., and Applied Science Laboratories, State College, Pa. Myo-phosphatidyl inositol was a gift of C. E. Ballou. These phospholipids were isolated from plant or animal tissue and had mixed fatty acid composition. The purity of the standards was checked by two-dimensional TLC in all cases.

All solvents used in this work were redistilled from glass stills and deoxygenated by bubbling nitrogen through them before use. Silica Gel HR, 20x20-cm TLC plates, plate spreader, and spreading template were obtained from Brinkman Instruments Inc., Westbury, N. Y. $MgSiO_2$ was obtained from Allegheny Chemical Company, Butler, N. J. Sephadex was purchased from Pharmacia Fine Chemicals Inc., New York, N. Y.

Optical densities of the phosphorus determinations were measured with a Zeiss PMQ-II spectrophotometer. Infrared spectra were

[1] Only whole plasma was investigated in this work. Previous investigators of the lipids in the circulation (excluding the formed elements of blood) have used whole plasma, defibrinogenated plasma, or serum. In this report the term plasma will be used exclusively; it refers to whole plasma with respect to the current work but can mean any of the derivatives of whole blood when referring to the work of previous investigators.

recorded with a Perkin-Elmer Model-521 grating spectrophotometer. Samples for infrared analysis were either dissolved in an appropriate solvent or mixed with KBr and pressed into a disk. Sealed liquid infrared cells had NaCl windows and path lengths of 0.1 or 1.0 mm.

Sampling

With two exceptions all of the animals used in this investigation were maintained in the laboratory by using standard animal husbandry methods; samples from horse and pig were obtained from local slaughter houses. Only mature animals were sampled. If an animal was large enough, sufficient blood for analysis was drawn from a single representative animal. The samples from guinea pig, rabbit, and rat represent pooled blood from six, two, and four animals respectively. Blood was drawn by venipuncture or cardiac puncture into sterile plastic blood bags for large animals or directly into plastic centrifuge tubes for the small animals. Heparin was used as an anticoagulant. The blood was cooled immediately to 0C and maintained at that temperature during processing. The cells were separated by centrifugation at 1570x*g* for 20 min in a refrigerated centrifuge. The plasma was removed by a volumetric pipette and transferred directly to the extraction flask.

Extraction Procedure

A measured volume of plasma was added slowly to cold methanol that was continuously stirred by a magnetic mixer. The flask was brought to volume with chloroform, and vigorous stirring was continued for 5 min. The final extraction volume was approximately 25:1 chloroform-methanol, 2:1 (v/v), to plasma. The solution was then filtered using a fast, prewashed filter paper (Whatman 41H, or Schleicher and Schuell Sharkskin), into a round-bottomed boiling flask. The solvent was removed by rotary evaporation at low temperature (<15C) and reduced nitrogen pressure. Before all the solvent was removed, the residue was transferred to a small graduated cylinder (5 ml) with chloroform-methanol 19:1 (v/v) and stored at –10C until further processing.

Sephadex Column Chromatography

Sephadex column chromatography, as described by Siakotos and Rouser (15), was used to removed nonlipid contaminants from the chloroform-methanol extract. The Sephadex (G-25, coarse) was washed, and the column was prepared as described (15) except that prewashing of the column was simplified by passing the elution solvent once through the column after packing and before applying the sample. Columns (2.5 x 10 cm) were fitted with Teflon needle-valve stopcocks to allow easy adjustment of the flow rate.

Three fractions were collected. Fraction I, 170 ml of 19:1 (v/v) chloroform-methanol, containing 5 ml of water per liter, contained all of the neutral lipids and phospholipids (organic-soluble phosphorus). Fraction II, 350 ml of 5 parts of 19:1 (v/v) chloroform-methanol and 1 part of acetic acid, which had 25 ml of water added to each liter, followed by 170 ml of 5 parts 9:1 (v/v) chloroform-methanol and 1 part of acetic acid, which had 42 ml of water added to each liter, contained any gangliosides present in the initial extract and traces of other organic substances. Fraction III, 350 ml of methanol-water 1:1 (v/v), contained nonlipid impurities in the initial extracts.

The first fraction was collected at a flow rate of 1 to 2 ml/min, and the following fractions at 3 to 4 ml/min. A column was allowed to stand at least 48 hr before re-use, and 500 ml of chloroform-methanol 19:1 (v/v), saturated with water, were passed through the column before the sample was applied.

The solvent was removed from Fraction I as described for the initial extract, and the sample was transferred to a glass-stoppered, graduated cylinder (5 ml) before all of the solvent was removed. At this time an aliquot was weighed to determine the total amount of lipid in this fraction. The remainder was stored as the solution at –10C until further analyses could be performed. Fractions II and III were not analyzed further except for a total weight determination of the material recovered in each fraction.

Two-Dimensional Thin-Layer Chromatography

Neutral lipids of the plasma were not analyzed in this investigation. The phospholipids were separated by two-dimensional TLC by using the methods of Rouser and coworkers (16, 17). TLC plates were spread with a 0.25-mm layer of Silica Gel HR, mixed with 10% $MgSiO_2$ by weight. Plates were activated for 20 min at 120C, then cooled in air for 30 min before applying the sample.

Samples and standards were spotted on the TLC plates from Lang-Levy micropipettes of appropriate volumes, 5 to 50 μl. Approximately 500 μg of phospholipid (20 μg P) were applied to the plate. Phospholipids in

TABLE I

Total Lipid and Percentage of Phospholipid in Plasma of Various Mammalian Species

Species	Volume of plasma extracted (ml)	Total lipid (Sephadex, F-1) (mg)	Total lipid (mg/ml plasma)	Percentage phospholipid[a] of total lipid
Cat	15.0	51.40	3.43	44.9
Cow	20.0	74.35	3.72	35.5
Dog	15.0	74.28	4.95	54.9
Goat	20.0	52.08	2.60	38.1
Guinea pig	15.0	14.96	1.00	21.6
Horse	25.0	72.40	2.90	43.8
Pig	10.0	51.68	5.17	33.1
Rabbit	10.0	26.80	2.68	45.9
Rat	16.4	51.05	3.18	38.5
Sheep	20.0	35.45	1.77	42.3

[a] Calculated as (mg lipid phosphorus × 25)/(mg total lipid).

the samples were separated by both pairs of solvent systems developed by Rouser and coworkers (16, 17). The first pair of solvents consisted of chloroform-methanol-ammonia 65:35:5 (v/v/v) in the first dimension, followed by chloroform-acetone-methanol-acetic-acid-water, 5:2:1:1:0.5 (v/v/v/v/v) in the second dimension. The second pair consisted of chloroform-methanol-water 65:35:4 (v/v/v), followed by butanol-acetic-acid-water 40:20:20 (v/v/v). The results in the two systems were the same within the experimental errors of the method, as determined by triplicate analyses of selected samples.

Spots were visualized by charring. The char reagent, 0.6% potassium dichromate in 55% by weight H_2SO_4, was sprayed on the TLC plate after the developing solvents had evaporated completely. Charring time was approximately 20 min at 180C in a forced-draft oven. Plates were photographed, as described previously (18), also viewed with UV light (3600 Å) to detect trace components.

Phospholipids were analyzed from the charred TLC plates by the technique of Rouser et al. (17). The spots were scraped off the plate into 5-ml, glass-stoppered tubes, and color reagents were added directly. The color was developed by methods reported previously (18). The total sample was also spotted on the plate and scraped off to check the recovery of phosphorus from the individual spots.

Additional plates spotted and chromatographed in an identical manner were sprayed with ninhydrin or Dragendorff's reagent to locate amino- and choline-containing lipids respectively. Still other plates were sprayed with distilled water, and the spots were outlined while the plates were wet. After the plates were dried in vacuo, the spots were scraped into sintered glass funnels so that the lipids could be eluted with an appropriate solvent. Lipids recovered from the TLC plates in this manner were further purified by chromatography on small Sephadex columns (19). The infrared (IR) spectra were then obtained on the lipids from individual TLC spots as indicated above.

RESULTS

The total lipid in mg/ml of plasma and the percentage of phospholipid of the total lipid are given in Table I for the 10 species investigated in this work. Total lipid ranged between 1.0 and 5.2 mg/ml for the guinea pig and pig respectively in this series. No lipemia was present in any of the plasma samples, and the plasma appeared normal in all other respects. All of the animals were mature and in good health. They were being fed ad libitum at the time of sampling so that all of the plasma may be considered to be from animals in the active absorption phase of digestion.

The dog had the highest percentage of circulating phospholipid, 54.9%, and the guinea pig had the lowest, only 21.6%. There was no discernible correlation between the percentage of phospholipid and the total amount of lipid in the plasma.

Fig. 1 gives representative infrared (IR) spectra of the major phospholipids of plasma isolated from TLC plates as described above. In all species the spectra were essentially identical with spectra obtained with standard reference compounds.

Sphingomyelin frequently gave rise to two distinct spots on the TLC plate. This phenomenon appears to be related exclusively to the degree of saturation in the acyl chains of the molecule, i.e., unsaturated molecules have separated from their saturated analogues. Infrared and gas chromatographic analyses of

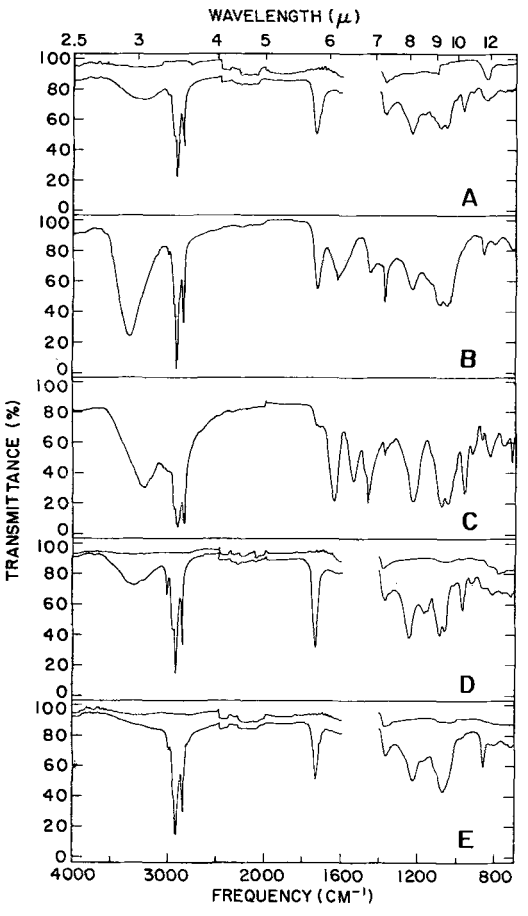

FIG. 1. The infrared spectra of the major lipids separated by TLC from the plasma of various mammals. Curve A: lysolecithin, 3.0 mg/ml in carbon disulfide solution. Curve B: phosphatidyl inositol from dog plasma, 66 μg in 50.0 mg KBr; the sharp absorption band at 1400 cm^{-1} is attributable to an impurity from the TLC absorbent. Curve C: sphingomyelin from cow plasma, 150 μg in 50.0 mg KBr. Curve D: lecithin from sheep plasma, 5.0 mg/ml in carbon disulfide solution. Curve E: phosphatidyl ethanolamine (may contain ethanolamine plasmalogens) from guinea pig plasma, 5 mg/ml in carbon disulfide solution.

both spots support this conclusion. Attempts to establish other chemical differences between the molecules to explain the phenomenon have been unsuccessful.

The phospholipid distribution in the plasma of each species is given in Table II. The main phospholipids in plasma are lecithin, lysolecithin, and sphingomyelin. Except in the guinea pig, rabbit, and rat, these choline-containing lipids account for 95% or more of the total phospholipids of plasma. Phosphatidyl ethanolamine and phosphatidyl inositol were the only other phospholipids detected. Phosphatidyl serine and phosphatidic acid, if present, were below the limits of detection in this investigation, <0.2% of the total phospholipid. No polyglycerol phospholipids (cardiolipin) or other lysophospholipids were detected. Traces of glycolipids were found on the TLC plates; the amounts were small in all cases, and no attempt was made to characterize the compounds further.

Lecithin was the main phospholipid in all species, varying from 82.6% in the dog to 55.7% in the guinea pig. Sphingomyelin varied by a factor of two, from 7.7% in the rat to 14.9% in the cow. Lysolecithin was more variable than either lecithin or sphingomyelin, by almost a factor of three, 7.8% in the dog to 22.7% in the rat.

The quantitative values reported in Table II are subject to experimental errors of ±5%, determined by triplicate TLC analyses for the major components present in amounts greater than 5% of the total phospholipids, whereas minor components can be in error as much as ±20%.

DISCUSSION

The analyses reported were performed on blood drawn from a single animal, usually in duplicate, for each of the species studied except where it was necessary to pool blood from several animals of a species as noted above. Although it is well known that plasma lipid distribution is influenced by diet (20-22) in different species, the phospholipids are less subject to dietary influences than are neutral lipids (23, 24). Furthermore elevation or reduction of the total amount of phospholipid present in plasma apparently does not alter the relative distribution of the individual phospholipid classes (25).

Vikrot (4), in a recent study on a large number of rats, found no significant variation in plasma phospholipid distribution among individual animals despite significant differences in the absolute amounts present. Högdahl and Vikrot (26), in a study of normal young women, reported similar results. Previous studies by Nelson and Freeman (27) and Phillips (28) on fractionated human serum lipoproteins has also established a lack of significant variation in phospholipid distribution among the various lipoprotein classes.

No attempt was made in this work to establish a normal range for the plasma phospholipids in the various species. However the

TABLE II

Plasma Phospholipid Composition in Various Mammalian Species[a]

Lipid class	Cat	Cow	Dog	Goat	Guinea pig	Horse	Pig	Rabbit	Rat	Sheep
Phosphatidyl ethanolamine[b]	1.3	1.2	1.3	0.8	21.7	3.3	1.9	6.7	1.3	1.0
Phosphatidyl inositol	0.3	1.1	1.9	0.3	0.8	0.3	2.8	5.5	4.3	0.5
Lecithin	69.3	74.5	82.6	77.7	55.7	74.9	69.9	60.8	63.9	71.7
Sphingomyelin	14.6	14.9	6.3	12.5	7.8	10.6	13.5	6.7	7.7	13.4
Lysolecithin	14.4	8.4	7.8	8.7	13.9	10.8	11.9	20.3	22.7	13.4

[a] Values represent average phosphorus determinations obtained by using duplicate analysis with two pairs of TLC solvents per sample; presented as weight percentage phosphorus of the total phospholipid phosphorus recovered for each phospholipid.

[b] Includes ethanolamine plasmalogen if present.

data do present an accurate analysis for a single animal in each species and, in view of the relative constancy of the plasma phospholipid distribution for members of a species, establish the general pattern of the plasma phospholipids in each species.

Limited data are available on phospholipid distribution in the plasma of various species. Taurog et al. (11) analyzed dog plasma phospholipids, and Sinclair (13) analyzed dog, horse, and cow. Rat plasma phospholipids have been studied by Newman et al. (6), Howard et al. (7), Lyman et al. (8), and Vikrot (9). All of these reports without exception agree that the major plasma phospholipids are lecithin, lysolecithin, and sphingomyelin although lysolecithin was not always separated as a distinct entity.

Dawson et al. (10) surveyed the largest number of species in a previous report. Their values agree well with those obtained in this work for pig, horse, cow, sheep, and goat although they did not separate lecithin from lysolecithin. They also concluded that phosphatidyl serine was absent from plasma.

The guinea pig appears to represent an exception to the trend established for the other species, with 21.7% phosphatidyl ethanolamine in its plasma phospholipids. Phosphatidyl ethanolamine in the rabbit (6.7%) was also higher than the average (1.5%) for the other species. The guinea pig however is even more unusual in that it appears to differ from the other species in this series in all of its plasma lipid parameters. It had the lowest total lipid (1.00 mg/ml) and lowest percentage of phospholipid (21.6%) of any species. In addition, the phospholipid distribution in guinea pig plasma is more similar to that reported in chickens by Ranney et al. (29) and turkeys by Sinclair (13) than to the mammals in this series. The physiological significance of these observations is unknown.

The data for the guinea pig were obtained on plasma pooled from six animals and should be representative of the plasma phospholipid distribution for this species. Further analyses will be necessary however to confirm these observations and establish the normal range in individual animals.

The ruminants, cow, goat, and sheep, have a typical plasma phospholipid composition, an observation that may have considerable physiological significance for these species because their erythrocytes exhibit a unique phospholipid pattern with absolutely no lecithin (Nelson, paper submitted for publication). Sheep cannot apparently exchange fatty acids between plasma and erythrocytes as has been observed in human beings and rats by Oliveira and Vaughan (30), presumably because lecithin is absent from their erythrocytes. Cow and goat may also lack this exchange reaction for similar reasons.

Although lysolecithin was established as a normal constituent of plasma only recently (31, 32), it appears to be ubiquitously distributed in mammalian plasmas in varying but significant amounts. There remains however some controversy about the in vivo existence of this substance since Glomest and co-workers (33, 34) have demonstrated a plasma enzyme that converts lecithin to lysolecithin while transferring a fatty acid to free cholesterol in vitro. In this work the blood was cooled to 0C immediately after drawing and kept at that temperature until extraction (as soon as possible after separating the cells); lysolecithin in appreciable amounts was still detected in all samples. Hence it would seem that lysolecithin is a true constituent of plasma in vivo.

ACKNOWLEDGMENTS

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The Incorporation of Ethanolamine into Ether-Containing Lipids in Rat Brain[1]

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ABSTRACT

After intracerebral injection of C^{14}-ethanolamine into rats, the ethanolamine phosphoglycerides were isolated and hydrolyzed with mild alkali and acid. The specific radioactivity of the diacyl, acyl alkenyl, and acyl alkyl glyceryl-3-phosphorylethanolamine, the diacyl and acyl alkenyl glyceryl-3-phosphorylcholine, and sphingomyelin was determined at 0.5, 2, 24, and 48 hours. The specific radioactivity-time relationships show that the ethanolamine plasmalogen is not a precursor for the glyceryl ether form but suggest that acyl alkyl glyceryl-3-phosphorylethanolamine is desaturated to form some of the acyl alkenyl glyceryl-3-phosphorylethanolamine. The radioactivity in the choline portion of the choline phospholipids was very low.

INTRODUCTION

ALTHOUGH THE PRESENCE of alkenyl and alkyl substituents in the same class of compounds in brain and other tissues has been recognized for some time, a biosynthetic relationship has been proposed but not demonstrated (1). Since brain tissue contains an unusually large amount of acyl alkenyl GPE[3] (2) and since acyl alkyl GPE accounts for 3.1% of the phospholipids of rat brain (3,4), this tissue was chosen for a study of possible relationships. A previous study (4) had demonstrated that a combination of chromatography of the intact lipids and hydrolytic procedures could be used to determine the specific activity of acyl alkenyl and acyl alkyl GPE.

METHODS

Four adult female albino rats (220 to 250 g) were used in these experiments. An injection of 1.0 μc (0.5 μmole) of (1,2–C^{14})–ethanolamine into the lateral ventricle was made under Avertin anesthesia, as described by Ansell and Spanner (5) and Davison and Gregson (6). Preparation of brain lipid extracts (7), the DEAE cellulose column separation of ethanolamine phosphoglycerides (4), and mild hydrolysis procedures (4) have been described previously. Samples containing 2 to 4 μmoles of either choline or ethanolamine phospholipids were subjected to hydrolysis. Portions of the first and second upper phases and of the last lower phase were taken for determination of phosphorus (4) and radioactivity contents. The upper-phase samples for radioactivity determination were dissolved in 5 ml of a xylene-dioxane-Cellosolve-naphthalene-PPO-POPOP mixture.[4] Portions of all lipid samples, including the last lower phase of the hydrolysis procedure, were taken to dryness in a counting vial before the addition of 5 ml of xylene containing 0.4% PPO. The radioactivity of all samples was determined by the use of a Packard Tri-Carb liquid scintillation spectrometer. The channels ratio method (8) was used to determine the efficiency of counting by comparison of the ratio of counts in two channels (50–1000 and 90–1000) with an experimentally determined graph of counting ratio against counting efficiency. Very little quenching was observed for any of these samples. The phosphorus content was taken as a measure of the molar content of phospholipid or phospholipid derivative for the purpose of calculating the specific radioactivity.

Thin-layer chromatography (TLC) was carried out (4) with 65:25:4 $CHCl_3$–MeOH–18 N NH_4OH (by vol). Areas of the TLC plates were assayed for radioactivity by scraping of the silica gel into vials and by the addition of 0.5 ml of water, one drop of 10% $Na_2S_2O_3$ (to reduce residual iodine to iodide), and 5 ml of the scintillator for aqueous solutions described above. Radioactivity recoveries were always approximately 100%. The distribution of radioactivity in the intact lipids was found to be the same by use of either TLC or DEAE column separation.

In some cases a silicic acid column was used for purification of phospholipids which

[1] Presented at the AOCS Meeting, Cincinnati, October 1965.
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[3] Abbreviations: GPE, glyceryl-3-phosphorylethanolamine; GPC, glyceryl-3-phosphorylcholine; TLC, thin-layer chromatography; dps, disintegrations per second.
[4] The composition of the mixture was 300 ml 2-ethoxy ethanol, 300 ml 1,4-dioxane, 100 ml xylene, 65 g naphthalene, 8 g PPO, and 0.4 g POPOP.

TABLE I
Chromatography on DEAE Cellulose of Rat-Brain Lipids Extracted 48 Hours After Intracerebral Injection of C^{14}-Ethanolamine [a]

Fraction	Eluant (v/v)	μmoles P	dps	dps/μmole
1	$CHCl_3$	0	0	—
2	"	0	9	—
3	9:1 $CHCl_3$-MeOH	0.1	3	—
4	"	13.7	54	3.9
5	"	4.2	14	3.3
6	"	0.2	10	—
7	"	0.1	6	—
8	7:3 $CHCl_3$-MeOH	7.5	1880	250
9	"	8.2	1810	220
10	"	1.3	218	170
11	"	0.2	24	—
12	"	0.2	19	—
13	MeOH	0.2	12	—
14	"	0.1	1	—
15	"	0.2	8	—
16	"	0.3	26	—
17	ammoniacal 4:1 $CHCl_3$-MeOH	0.4	22	—
18	"	0.3	14	—
19	"	8.4	22	2.6
20	"	0.9	4	—
21	"	0.2	0	—

[a] The column was loaded with 44.4 μmoles of lipid P. The recovery was 105%.

were stable to hydrolysis. Columns, 1 cm diam., were packed with 1.0 g of silicic acid as a slurry in chloroform to a height of 1.9 cm. From 1–2 μmoles of phospholipid were present in the sample, which was applied to the column in chloroform. Four-ml portions of solvents were used for elution. Fraction A was eluted with chloroform, B with 7:3 (v/v) chloroform-methanol, C with 2:3 (v/v) chloroform-methanol, and D with methanol.

RESULTS

The ethanolamine phosphoglycerides were well separated from other brain lipids by chromatography on a column of DEAE cellulose in the acetate form (Table I). Major fractions were identified by TLC. Cholesterol was found in fractions 2 and 3, cerebrosides in 2–4, choline lipids in 4 and 5, ethanolamine lipids in 8–10, and acidic lipids (including serine phosphoglycerides) in 19.

The distribution of radioactivity which was found in column fractions after four time periods is summarized in Table II. The bulk of the radioactivity was found in the ethanolamine phosphoglycerides. A small but significant amount of radioactivity was in unidentified neutral lipids eluted by chloroform. The choline lipid fraction contained a small amount of radioactivity although the choline lipids were completely separated from the ethanolamine lipids. The compounds eluted after the ethanolamine lipids were not characterized because of their low level of radioactivity. The results obtained by TLC of the intact lipids agreed with those from column separations. Ethanolamine and choline lipid fractions were pooled separately on the basis of phosphorus and radioactivity determination.

The pooled ethanolamine phospholipid fractions were subjected to hydrolysis by mild alkali and mild acid. The results of radio-

TABLE II
Distribution of Radioactivity in DEAE Cellulose Column Fraction of Rat-Brain Lipids at Various Times After Intracerebral Injection of C^{14}-Ethanolamine

Eluant (v/v) and Eluate	Time, Hours: 0.5	2	24	48
	Radioactivity, dps per brain			
$CHCl_3$, nonpolar lipids	2	10	20	22
9:1 $CHCl_3$-MeOH, choline lipids	21	45	163	218
7:3 $CHCl_3$-MeOH, ethanolamine lipids	600	3069	11445	9878
MeOH, water-soluble compounds	263 [a]	100	218	118
Ammoniacal 4:1 $CHCl_3$-MeOH, acidic lipids	25 [b]	14 [b]	352	155

[a] Probably includes ethanolamine not removed by a single wash of the lipid extract.
[b] Elution of these fractions was not complete.

TABLE III
Results of Mild Hydrolysis of Rat-Brain Ethanolamine Phospholipids Obtained by DEAE Cellulose Chromatography

	Time, Hours			
	0.5	2	24	48
	Percentage of the ethanolamine phospholipid fraction			
Diacyl GPE	45	42	41	43
Acyl alkenyl GPE	48	50	53	47
Acyl alkyl GPE	7	8	6	9

activity and phosphorus assays are given in Tables III and IV. The ethanolamine lipids from the 48–hour experiment which were stable to hydrolysis were applied to a silicic acid column. More than 90% of the phosphorus and radioactivity was recovered in fractions B and C. A spot representing 8% of the stable ethanolamine lipids with the same R_f value as intact ethanolamine phospholipids was found by TLC of fraction B. It had a specific radioactivity of 120 dps per μmole. The TLC spots representing alkyl GPE from fractions B and C had a specific radioactivity of 240.

The pooled choline phospholipid fractions were also examined by the hydrolysis method (4). Tables V and VI report the results of phosphorus and radioactivity assays. The intact choline phospholipid fraction from the 0.5–hr experiment had a specific radioactivity of 0.6 dps per μmole P. It was not examined further. Because of the relatively large amount of radioactivity in the stable choline lipids from the 24–hr and 48–hr experiments, the specific radioactivity of sphingomyelin was measured by other methods. The intact choline lipids from the 24–hr experiment were separated by TLC. No radioactivity was detected in sphingomyelin. The lecithin had a specific radioactivity of 4.7. Two samples of choline lipids which were stable to hydrolysis were purified on silicic acid columns. Pooled fractions C and D contained phospholipids with a specific radioactivity of 1.1 and 1.0 dps per μmole P from the 24– and 48–hr experiments respectively. TLC was also used to examine the last lower-phase lipids from the 24–hr experiment. The phosphorus distribution was 10% in lysolecithin (probably alkyl GPC) and 90% in sphingomyelin. No radioactivity was found at the origin or associated with the lysolecithin. The sphingomyelin had a specific radioactivity of 1.6 dps per μmole P and accounted for 6% of the radioactivity in the fraction. The cerebroside area contained 37%, and the solvent front contained 57% of the total radioactivity originally present in the pooled choline phospholipid fraction.

DISCUSSION

The best available evidence has indicated that ethanolamine phospholipids are formed in the brain by the condensation of the appropriate disubstituted glycerol with cytidine diphosphate ethanolamine to form the phospholipid and cytidine monophosphate (9). Constant radioactivity ratios between each type of ethanolamine phospholipid would be expected if cytidine diphosphate ethanolamine were the immediate precursor for each. On the basis of the present results and others (5, 10–12) the cytidine diphosphate ethanolamine pathway may not be the only or even the major pathway for the in vivo labelling of acyl alkenyl GPE under these conditions. The differences in the radioactivity ratios of the diacyl GPE and

TABLE IV
Specific Radioactivity of Ethanolamine Phospholipid Types at Various Times After Intracerebral Injection of C^{14}-Ethanolamine

	Time, Hours			
	0.5	2	24	48
	Specific radioactivity, dps per μmole			
Diacyl GPE	40	139	406	295
Acyl alkenyl GPE	4.5	29	193	174
Acyl alkyl GPE	24.5	66	360	196
Total GPE	19.9	81	298	219
	Ratio of specific radioactivities			
$\frac{\text{Acyl alkyl GPE}}{\text{Acyl alkenyl GPE}}$	5.3	2.3	1.9	1.1
$\frac{\text{Acyl alkyl GPE}}{\text{Diacyl GPE}}$	0.61	0.47	0.89	0.66

TABLE V
Results of Mild Hydrolysis of Rat-Brain Choline Phospholipids Obtained by DEAE Cellulose Chromatography

	Time, Hours		
	2	24	48
	Percentage of the choline phospholipid fraction		
Diacyl GPC	80	81	80
Acyl alkenyl GPC	3	3	3
Sphingomyelin [a]	16	16	17

[a] Probably includes some acyl alkyl GPC. (See the text for details.)

the acyl alkyl GPE are not sufficient to rule out the cytidine pathway for biosynthesis of acyl alkyl GPE. It was reported (13) that this compound is synthesized at an unusually high rate from cytidine diphosphate (C^{14}) ethanolamine by brain tissue in vitro. It is now certain that the unusually high specific radioactivity was caused by an unknown compound present in the synthetic labelled nucleotide (14).

The present results certainly suggest that acyl alkyl GPE gives rise to some acyl alkenyl GPE. The plasmalogen form cannot be a precursor of the glyceryl ether form as previously suggested (1) because the plasmalogen form had a lower specific radioactivity at all times examined (0.5 to 48 hr). The specific radioactivity–time relationships observed in this study resemble those found by Thompson (15) for total phospholipids of the slug (*Arion ater*). We must mention however that in neither study was a crossover point found for specific activities of alkyl and alkenyl substituted phospholipids, as would be expected if the alkenyl compound were the only product formed from the alkyl compound (see the discussions of precursor–product relationships by Zilversmit et al. (16) and Reiner (17)). Some of the early steps in the pathway proposed by Thompson (15) for biosynthesis of acyl alkyl phospholipids in the slug do not appear to agree with data obtained by Malins (18) with dogfish (*Squalus acanthias*) liver.

As shown in Table VI, the amount of radioactivity found in the GPC portions of diacyl and acyl alkenyl GPC was very low. The absence of any significant formation of choline from ethanolamine in the brain (19,20) is confirmed by these data.

A substantial portion of the radioactivity in the choline lipid fraction, 85% at 24 hr, was found in the lipids which were stable to hydrolysis. Most of this radioactivity was separated from sphingomyelin by chromatography and appeared to be present in cerebrosides and in the fatty acids and aldehydes produced by hydrolysis. At 24 hr the specific radioactivity values found for purified sphingomyelin were 0, 1.1, and 1.6 dps per μmole P by different methods. For comparison, intact diacyl GPC gave a value of 4.7 by TLC and the GPC portion from diacyl GPC gave a value of 0.7. The results with choline lipids suggest that a small portion of the injected ethanolamine was metabolized to acetate (21) and then to fatty acids.

The phospholipid composition found for these rat-brain extracts is the same as reported previously (4). The specific radioactivities of the diacyl and acyl alkenyl GPE and GPC types agree well with those obtained by Ansell

TABLE VI
Specific Radioactivity of Choline Phospholipid Types at Various Times After Intracerebral Injection of C^{14}-Ethanolamine

	Time, Hours		
	2	24	48
	Specific Radioactivity, dps per μmole P		
Diacyl GPC	0.4	0.7	2.1
Acyl alkenyl GPC	2	1	4
Sphingomyelin [a]	4	22	18
	Ratio of specific radioactivities		
$\frac{\text{Diacyl GPC}}{\text{Diacyl GPE}}$	0.003	0.002	0.007
$\frac{\text{Acyl alkenyl GPC}}{\text{Acyl alkenyl GPE}}$	0.07	0.005	0.02

[a] These values are high, as explained in the text. (See also Table V, footnote a.)

TABLE VII
Relative Specific Radioactivities of Rat-Brain Ethanolamine Phospholipids, Using Ethanolamine and Phosphate Precursors

	Phosphate (22) [a]	Ethanolamine [b]
Alkali-stable, 24 hr / Alkali-labile	0.48	0.51
Alkali-stable, 48 hr / Alkali-labile	0.69	0.60

[a] These values were calculated by division of the value for microsomal phosphatidal ethanolamine by the value for microsomal phosphatidyl ethanolamine at the indicated times.

[b] The calculated specific radioactivity for the sum of acyl alkenyl GPE and acyl alkyl GPE was divided by the value for diacyl GPE for the purpose of comparing the two sets of data.

and Spanner (5), maintaining the same injection conditions but using the method of mild alkaline and acid hydrolysis, followed by paper chromatographic separation of the water-soluble moieties. Mandel and Nussbaum used a similar technique, but with a shorter alkaline hydrolysis, to determine the incorporation of P^{32}–phosphate into the major lipids of rat-brain subcellular fractions (22). A comparison of the specific radioactivities of two fractions of ethanolamine phospholipids is given in Table VII. The agreement is very good.

A possible complication in interpretation of the data arises if the myelin in the rat brain contains a disproportionate share of any of the three types of ethanolamine phosphoglycerides. The ethanolamine plasmalogen appears to be concentrated in the myelin of rat brain (23,24) but not in that of the ox (25). However Mandel and Nussbaum (22) found similar curves for specific radioactivity against time for rat-brain myelin, microsomes, and mitochondria for all of the major lipid types. No information has been reported on the subcellular distribution of acyl alkyl GPE in brain tissue.

Possible errors which could arise from analysis by the mild hydrolysis procedures include premature release of GPE from acyl alkenyl GPE into the aqueous phase from alkaline hydrolysis and a carryover of alkenyl GPE into the last lower phase because of cyclic acetal formation. If either or both occurred, the specific radioactivity assigned to acyl alkenyl GPE would still be correct while the values for diacyl GPE and for acyl alkyl GPE would be depressed. None of those situations would change the conclusions of this report. The former possibility has been discussed (4). The latter possibility is unlikely because a detailed examination of the stable ethanolamine lipids from the 48-hour experiment gave TLC spots with the correct R_f value for alkyl GPE and with a specific radioactivity in good agreement with the value found for the total stable lipids.

These results are the first reported examination of metabolic relationships between plasmalogens and the corresponding glyceryl ether phospholipids in mammalian brain in vivo. It is proposed that acyl alkenyl GPE may be formed by desaturation of acyl alkyl GPE in rat brain.

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Determination of Plasmalogens after Treating with a 2,4-Dinitrophenylhydrazine-Phosphoric Acid Reagent[1]

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ABSTRACT

A method for the quantitative determination of plasmalogens is described. Glycerophosphatide fractions from animal and plant sources were treated with a 2,4-dinitrophenylhydrazine-phosphoric acid reagent. The plasmalogen content was estimated by two different procedures after a single treatment with reagent: a) by determining the phosphorus content of diacyl phospholipid and (2-acyl) lysophospholipid separated by thin-layer chromatography, b) by spectrophotometric determination of the 2,4-dinitrophenylhydrazones and phosphorus assay of the original untreated sample. The total plasmalogen content of an unfractionated extract may be determined easily by the latter procedure.

The method described gives results comparable with those from the p-nitrophenylhydrazine method and iodination procedure. The usefulness of this 2,4-dinitrophenylhydrazine-phosphoric acid treatment for further structural analyses of plasmalogens is discussed.

INTRODUCTION

OF THE MANY PROCEDURES reported for plasmalogen determination, the most widely used are the fuchsin-sulfurous acid (5, 13, 20, 28, 29) and p-nitrophenylhydrazine methods (19, 21, 31) for plasmalogen-bound aldehyde determination and the iodination procedure which is specific for the vinyl ether bond (7, 15, 30).

The formation of 2,4-dinitrophenylhydrazones (DNP hydrazones) on a Celite column impregnated with 2,4-dinitrophenylhydrazine (DNP hydrazine) and phosphoric acid has been used to isolate carbonyl compounds from reverted or oxidized fats and oils (14, 18, 27). Katz and Keeney (10) described a spectrophotometric procedure for determination of plasmalogen-bound aldehydes which ultilized simultaneous methanolysis of the acyl phospholipid component and formation of DNP hydrazones of the fatty aldehydes.

It was thought that, if only the fatty aldehydes are cleaved from a plasmalogen by an acidic DNP hydrazine reagent to form DNP hydrazones, leaving the diacyl phospholipid intact, the determination of the lysocompound formed from the plasmalogen could be used for estimating the plasmalogen content of the phospholipid fraction concerned. This paper describes a method for determining the plasmalogen content of a phospholipid by using a DNP hydrazine-phosphoric acid reagent. The plasmalogen content was estimated by determining the DNP hydrazones of plasmalogen-bound aldehydes and by phosphorus assay of the (2-acyl) lysophospholipid and diacyl phospholipid components after thin-layer chromatography.

When this study was in completion, Owens (16) reported a two-dimensional thin-layer chromatographic procedure for the estimation of plasmalogens. His method was based also on the specific hydrolysis of plasmalogens to (2-acyl) lysophospholipid by the use of a mercuric chloride spray reagent.

EXPERIMENTAL PROCEDURE

Reagents

DNP hydrazine (Eastman Organic Chemicals) was purified as described by Katz and Keeney (10).

Carbonyl-free hexane and chloroform were prepared by the method of Schwartz and Parks (26). Carbonyl-free methanol was prepared as described by Katz and Keeney (10).

The reagent for cleaving the vinyl ether linkage of plasmalogens was prepared from purified DNP hydrazine (300 mg), dissolved in 12 ml of 85% H_3PO_4 by grinding in a mortar. Distilled water (8 ml) was added to the clear yellow solution, and the precipitated DNP hydrazine was redissolved by grinding. The final concentration of this reagent was 15 mg of DNP hydrazine per milliliter of 51% H_3PO_4. This reagent was freshly prepared bi-weekly and stored in a dark bottle.

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Separation of Lipids

Total lipids were extracted from fresh hog and beef brains and beef liver by the method of Bligh and Dyer (1), and the extract was washed according to the procedure developed by Folch et al. (6). Ethanolamine glycerophosphatide (EGP) of brains was isolated by column chromatography on DEAE cellulose (24), and choline glycerophosphatide (CGP) was separated on a silicic acid-silicate-water column (23) from the first fraction of DEAE cellulose column chromatography.

A commercial soybean phospholipid sample (Centrolex P, Central Soya, Chicago, Ill.) contained EGP and CGP as major phospholipids, as shown by thin-layer chromatography (TLC). The two phospholipids were separated by silicic acid (Mallinckrodt) column chromatography by the scheme used by Kuchmak and Dugan (12). As the two phospholipid fractions thus isolated showed nonlipid contaminants on TLC, the two fractions were cleaned by passage through separate DEAE cellulose columns (15 cm, 2.2 cm I.D.) CGP was eluted from one column with chloroform-methanol (9:1) and EGP from the other with chloroform-methanol (7:3). All solvent mixtures used in the study were prepared in v/v ratios unless otherwise specified.

The final EGP and CGP fractions from brains and the soybean phospholipid sample were pure as monitored by TLC.

Reaction of Lipids with the DNP Hydrazine-H_3PO_4 Reagent

A stream of nitrogen was used to remove the solvent from an aliquot of a solution containing a sample (up to 5 μmoles of phosphorus) in a 50-ml centrifuge tube with glass stopper. The lipid was then redissolved in 0.2 ml of carbonyl-free chloroform-methanol (1:1); 1 ml of the DNP hydrazine-H_3PO_4 was added, and the contents were shaken thoroughly. Then the centrifuge tube was stoppered under nitrogen and allowed to stand for one and one-half hours, with occasional shaking, in a dark cabinet at room temperature.

Distilled water (10 ml) was added to the reaction mixture. The DNP hydrazones, (2-acyl) lysophospholipid, and diacyl phospholipid were extracted with three 5 ml-portions of carbonyl-free hexane. Centrifuging was used when necessary to break emulsions or remove the excess precipitate of DNP hydrazine (reagent) from the hexane layer. The extract was washed with distilled water until the washing water became colorless. Three washes with 10 ml of water each were usually adequate. The washed hexane solution was dried over anhydrous sodium sulfate before it was made up to volume. The absorbance of the hexane extract was measured, against a blank prepared without lipid, at 338 mμ on a Beckman DU spectrophotometer. The concentration of DNP hydrazones was calculated from a molar absorptivity of 21,500 (9).

TLC and Phosphorus Assay of TLC Spots.

Chromatoplates were prepared with the Desaga applicator by using a slurry of Silica Gel G (Merck) in distilled water, 1:2 (w/v).

For TLC of the DNP hydrazones, diacyl phospholipid, and (2-acyl) lysocompound the hexane extract was concentrated under a stream of nitrogen and the chromatogram was developed with chloroform-methanol-ammonium hydroxide (conc.) (75:25:4). The DNP hydrazones traveled nearly to the solvent front and were observed without any spray. The lysocompounds and diacyl phospholipids were made visible by spraying with ninhydrin (0.2% ninhydrin in n-butanol/aqueous acetic acid 10%, 95:5), the sensitive molybdenum spray developed by Dittmer and Lester (4) and sulfuric acid (50%), followed by charring.

The plates were exposed to iodine vapor, and each spot was outlined for phosphorus assay of the phospholipid component in each of the spots. After the iodine vapor had evaporated from the plate, the phosphorus contents of TLC spots of the phospholipids were determined by Parker's procedure (17) after aspirating the silica gel containing each of the spots into a flask and digesting with 1 ml of perchloric acid (70%) at a mild heat setting of an electrically heated digestion rack (22,25).

A basic developing solvent mixture, chloroform-methanol-ammonium hydroxide (75:25:4), was most effective for TLC of the hexane extract since any residual phosphoric acid was neutralized at the origin and did not streak as it did when nonbasic solvents were used.

Column Chromatography of the Hexane Extract

A silicic acid column was prepared in chloroform-methanol (1:1), for column separation of the DNP hydrazones, diacyl phospholipid, and (2-acyl) lysocompound in the extract. The hexane extract was concentrated to 0.2 ml under a nitrogen atmosphere, and 0.2 ml of chloroform was added to this before application to the column. The DNP hydrazones were eluted with chloroform. If desired, the phospholipids may be eluted with mixtures of chloroform and methanol: 3:1 for phosphatidyl

ethanolamine, 3:2 for phosphatidyl choline, 1:3 for (2-acyl) lysophosphatidyl ethanolamine and methanol for (2-acyl) lysophosphatidyl choline.

RESULTS

Comparison of Methods

The plasmalogen contents of EGP and CGP fractions from hog brain and the soybean phospholipid sample were determined by two different procedures after a single treatment with the DNP hydrazine-H_3PO_4 reagent as follows: a) by determining the phosphorus content of diacyl phospholipid and (2-acyl) lysophospholipid separated by TLC, b) by spectrophotometric determination of the DNP hydrazones and phosphorus assay of the original untreated glycerophosphatide.

Fig. 1 shows the TLC chromatograms of the untreated and DNP hydrazine-H_3PO_4 treated EGP of hog brain and CGP of the soybean phospholipid sample. Usually components of spots from two or three chromatograms were pooled for phosphorus assay when the glycerophosphatide contained a very small amount of plasmalogen. The DNP hydrazone measurement was also carried out with samples shaken with saturated sodium bisulfite solution to remove the free aldehydes which resulted from lipid peroxidation. The sodium bisulfite-treated samples were thoroughly washed with distilled water and dried over anhydrous sodium sulfate before the DNP hydrazine-H_3PO_4 treatment.

The results by both procedures are shown in Table I. The DNP hydrazone determination, without removing free aldehydes, gave higher apparent values of plasmalogen content for all phospholipid fractions tested. The differences between this method and the phosphorus determination procedure varied between 1% and 5%, depending on the phospholipid, whereas the samples with free aldehyde-removed showed nearly the same results as those based on the phosphorus determination.

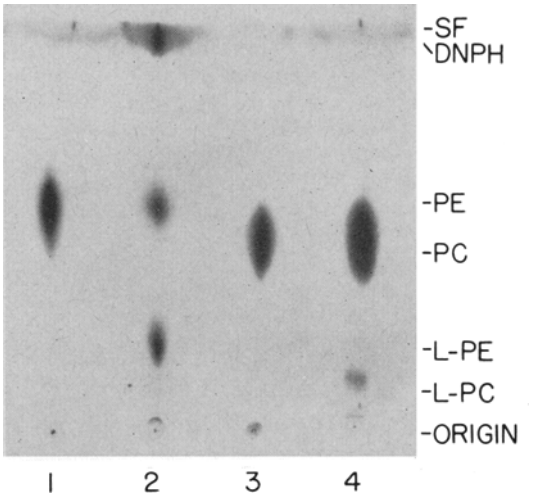

FIG. 1. TLC of the untreated and DNP hydrazine-H_3PO_4-treated glycerophosphatides. Adsorbent: Silica Gel G; solvent system: chloroform-methanol-ammonium hydroxide (75:25:4); indicator: 50% sulfuric acid spray followed by charring. 1. Untreated ethanolamine glycerophosphatide from hog brain; 2. hexane extract of the treated ethanolamine glycerophosphatide from hog brain; 3. untreated choline glycerophosphatide from the soybean phospholipid sample; 4. hexane extract of the treated choline glycerophosphatide from the soybean phospholipid sample. SF: solvent front; DNPH: 2,4-dinitrophenylhydrazones (for Samples 2 and 4); PE: phosphatidyl ethanolamine plus phosphatidal ethanolamine for Sample 1 and phosphatidyl ethanolamine for Sample 2; PC: phosphatidyl choline plus phosphatidal choline for Sample 3 and phosphatidyl choline for Sample 4; L-PE: (2-acyl) lysophosphatidyl ethanolamine; L-PC: (2-acyl) lysophosphatidyl choline.

TABLE I

Comparison of Plasmalogen Content Determined by Phosphorus Assay with That by DNP hydrazone after Treatment with 2,4-DNP hydrazine-H_3PO_4[a]

Lipid source	Phospholipid fraction[b]	% Plasmalogen: P Assay procedure	% Plasmalogen: DNP hydrazone determination: After $NaHSO_3$ treatment	% Plasmalogen: DNP hydrazone determination: Prior to $NaHSO_3$ treatment
Hog brain	EGP	56	55	61
	CGP	8	9	12
Soybean	EGP	4	4	7
	CGP	8	—	9

[a] Each figure represents the average of two analyses.

[b] EGP: ethanolamine glycerophosphatide; CGP: choline glycerophosphatide.

TABLE II

Plasmalogen Content of Ethanolamine Glycerophosphatide Fraction from Hog Brain as Determined by the DNP hydrazine-H_3PO_4 Treatment and Acetic Acid Incubation Method[a]

Method	Trial	% Plasmalogen
DNP hydrazine-H_3PO_4	a	55
	b	55
	c	57
Acetic acid (90%)	a	49
	b	53
	c	48

[a] Percentage of plasmalogen estimated by P assay of phophatidyl ethanolamine and (2-acyl) lysophosphatidyl ethanolamine after TLC.

TABLE III

Comparison of the 2,4-Dinitrophenylhydrazine-H_3PO_4 Method (2,4-DNPH), p-Nitrophenyl-Hydrazine Method (p-NPH), and Iodination Procedures for Plasmalogen Content[a,b]

Lipid[c]	Moles of Plasmalogen/l lipid solution		
	2,4-DNPH[d]	p-NPH	I_2 uptake
EGP (beef brain)	$(2.170 \pm 0.005) \times 10^{-3}$	$(2.158 \pm 0.008) \times 10^{-3}$	$(2.164 \pm 0.014) \times 10^{-3}$
CGP (beef brain)	$(0.603 \pm 0.010) \times 10^{-4}$	$(0.560 \pm 0.020) \times 10^{-4}$	$(0.566 \pm 0.170) \times 10^{-4}$
Total beef liver lipids	$(8.618 \pm 0.031) \times 10^{-4}$	$(8.460 \pm 0.051) \times 10^{-4}$	$(8.570 \pm 0.031) \times 10^{-4}$
Total beef brain lipids	$(2.256 \pm 0.031) \times 10^{-4}$	$(2.205 \pm 0.046) \times 10^{-4}$	$(2.197 \pm 0.037) \times 10^{-4}$

[a] Each figure represents the results of three analyses.

[b] The final concentration of each lipid preparation was not the same, but the same volume of aliquot was used for the three methods with a given preparation.

[c] EGP: ethanolamine glycerophosphatide; CGP: choline glycerophosphatide.

[d] Spectrophotometric determination of 2,4-dinitrophenylhydrazones.

Completeness of Cleavage of Vinyl Ether Linkage by the DNP Hydrazine-H_3PO_4 Reagent

The recovery of plasmalogen-bound aldehydes by the DNP hydrazine-H_3PO_4 reagent was compared with the acetic acid incubation method described by Gray (8). To cleave vinyl ether linkage of plasmalogen by acetic acid, EGP of hog brain (10 μmoles or less) was incubated with 2 ml of 90% acetic acid at 38C for 18 hr. The free aldehydes, phosphatidyl ethanolamine, and (2-acyl) lysophosphatidyl ethanolamine were extracted as described by Gray. Table II shows the results of three separate trials for each treatment. The percentage of plasmalogen of the EGP fraction was slightly higher as estimated by the DNP hydrazine-H_3PO_4 treatment than by the 90% acetic acid incubation. If 100% cleavage of the vinyl ether linkages of phosphatidal ethanolamine by the H_3PO_4-containing reagent is assumed, the acetic acid cleaved about 90% of the linkages.

To test any possible splitting of the ester linkage in phospholipids by the DNP hydrazine-H_3PO_4 reagent, pure phosphatidyl ethanolamine and phosphatidyl choline (diacyl moieties) were separated by silicic acid column chromatography after reacting EGP and CGP of hog brain with the reagent and extracting the resulting components with hexane. Each solvent-free diacyl phospholipid was again reacted with the reagent, and the filtered aqueous phase (filtered to remove the excess DNP hydrazine) and the hexane phase were chromatographed on TLC plates of Silica Gel G after concentrating to a small volume (0.1-0.2 ml) under a stream of nitrogen or in vacuo. No water-soluble glycerylphosphoryl ethanolamine or choline was detected in the aqueous phase, and the hexane phase showed only phosphatidyl choline but no corresponding lysophospholipids, indicating that the DNP hydrazine-H_3PO_4 treatment does not split the acyl ester linkage of the phospholipids.

Comparison with Other Methods

The spectrophotometric procedure of the present method was compared with the p-nitrophenylhydrazine method of Wittenberg et al. (31) and the iodination procedure of Williams et al. (30) by using separated phospholipid fractions and total lipid extracts of beef brain and liver. The results are presented in Table III. Plasmalogen contents by the three methods were in close agreement for each lipid preparation, but the DNP hydrazine-H_3PO_4 method gave better reproducibility and slightly higher results.

The TLC procedure of this DNP hydrazine-H_3PO_4 method was also compared with the two dimensional TLC method of Owens (16). The EGP of hog brain, containing $(56 \pm 1)\%$ of plasmalogen as estimated by the former method (Table II), showed $(53 \pm 2)\%$ of plasmalogen in three analyses by the latter.

DISCUSSION

The observation that ethanolamine plasmalogen is highly predominant over choline plasmalogen in hog brains is in accord with other results obtained with brain tissues of animals (2,3). The presence of plasmalogens, although in small amounts, in plant products such as soybeans and other legumes has been reported (11), but there was a question as to whether or not the results could be ascribed to artifacts or defects in methodology. As demonstrated in Table I, the soybean phospholipid sample which was used contained ethanolamine plasmalogen and choline plasmalogen at levels of less than 10% of the corresponding glycerophosphatide.

The present method has some advantages. The method seems to have better reproducibility than other methods with which it was

compared. The plasmalogen content of a separated glycerophosphatide fraction can be determined by two different ways after single treatment with the reagent. The TLC separation and phosphorus determination procedure may be better than the DNP hydrazone measurement, if the glycerophosphatide fraction is not very fresh, because the aldehydes from lipid peroxidation would also be converted to DNP hydrazones by the treatment. If the DNP hydrazone measurement is still preferred, the free aldehyde should be removed by treating the sample with saturated sodium bisulfite solution before reacting with the DNP hydrazine-H_3PO_4 reagent. The method is useful for further analyses of fractionated phospholipids. It facilitates the complete structural analysis of a plasmalogen. The DNP hydrazones can be converted to dimethyl acetals, and the fatty aldehydes at the α' position can be identified by gas chromatographic analysis of the dimethyl acetals. The fatty acids at the β position can be identified by gas chromatography of methyl esters of the fatty acids in the (2-acyl) lysocompound.

The method described in this paper and the procedure of Owens (16) have a similarity. In both methods the plasmalogen content of a glycerophosphatide can be estimated by phosphorus determination of the diacyl phospholipid and (2-acyl) lysophospholipid formed by the treatments. When the percentage of plasmalogen, as a whole, of an unfractionated lipid extract or total phospholipid sample is to be estimated without concerning the individual plasmalogens, the spectrophotometric DNP hydrazone measurement procedure of the present method is much simpler. When the procedure of Owens is used for this purpose, phosphorus analyses of individual diacyl phospholipids and (2-acyl) lysophospholipids should still be made. If the plasmalogen content of individual glycerophosphatides in an unfractionated extract is to be determined with no attempt for further structural analyses of each plasmalogen, the Owens' procedure may be advantageous since it eliminates the necessity of prior TLC or column separation of glycerophosphatides.

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Determination of Petroselinic Acid by Microreactor Chromatography

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ABSTRACT

The microreactor-ozonolysis technique was applied to the quantitative determination of the relative amounts of petroselinic and oleic acids in seven Umbelliferae seed oils. The operation was easy and rapid. Results were excellent when the method was tested on ester mixtures of known composition. When used on esters prepared from Umbelliferae seed oils, the method gave results comparable with those found by another procedure, also described, which combined thin-layer chromatography with either gas-liquid chromatography or ultraviolet spectrophotometry.

INTRODUCTION

In studying the composition of seed oils from many species of plants, investigation of the family Umbelliferae has been deferred because no rapid, reliable method was available for determining petroselinic acid in the presence of oleic acid. Older methods which utilized ester distillation and lead-salt separations (1) were recognized as containing certain errors (2). More recently oxidation with permanganate-periodate and analysis of the oxidation products by gas-liquid chromatography (GLC) (3) or by column chromatography (4) have proved useful. Ozonolysis, followed by GLC, appears to constitute a further improvement (5).

Ozonolysis in the microreactor apparatus (MRA), developed by Davison and Dutton (6) for determining the location of double bonds in fatty acids, has now been successfully applied to the quantitative determination of petroselinic acid in seed oils of the Umbelliferae. Results are compared with another method, developed in this laboratory, utilizing thin-layer chromatography (TLC) and either GLC or ultraviolet spectrophotometry.

METHODS

Oils were extracted from the seed or seed plus pericarp with petroleum ether (bp 30-60C) (7). The oil was saponified, and unsaponifiable matter was removed by the usual procedures (8). Free acids were recovered and esterified with diazomethane or with 5% HCl-methanol (w/v).

Microreactor

Ozonolysis of methyl esters and pyrolysis of the ozonides were accomplished in an MRA, and products were injected directly into the column for GLC as previously described (6). The 30x1/4-in. aluminum column was packed with 20% poly(ester-acetal) (9) or 30% diethylene glycol succinate on 40-60 mesh Chromsorb W.

The C_6 and C_9 aldehyde-ester ozonolysis products of methyl oleate and methyl petroselinate were used for quantitation since these products elute from the chromatographic column as single components whereas the C_{12} and C_9 aldehydes often are superimposed on other ozonolysis products and require correction factors.

The suitability of the procedure was demonstrated by analysis of mixtures containing known amounts of methyl petroselinate and methyl oleate (A, B, C, Table I), which were prepared from Hormel standard samples, and of two mixtures containing methyl palmitate and methyl linoleate in addition (D, E). The amount of C_9 aldehyde-ester derived from methyl linoleate was considered in calculating the amount of methyl oleate in samples D and E.

A similar correction for methyl linoleate was made for the Umbelliferae seed oil methyl esters. In a second analysis of the Umbelliferae esters and of standards D and E, the 18:1 fractions were isolated by preparative GLC in an aerograph A-700 "Autoprep," equipped with a 10 ftx1/4 in. copper column packed with 15% LAC-2-R 446 on 40-60 mesh Celite 545 run isothermally at 180C. A 12-μl injection yielded more than enough 18:1 (approximately 6 mg) for analysis with the MRA. The 18:2 fraction from *Daucus guttatus* esters was isolated in the same manner as the 18:1 fraction.

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TABLE I

Determination of Methyl Petroselinate in Standard Mixtures and in Mixed Esters from Seed Oils

Sample	Methyl Petroselinate in Total 18:1[a]			
	Wt %	TLC %	MRA %	MRA-Prep. GLC %
Standard A	87.8		84	
Standard B	50.7		52	
Standard C	10.6		11	
Standard D[b]	74.2		76	76
Standard E[c]	25.4		27	26
Actinolema macrolema Boiss.		62[d]	70	62
Bupleurum croceum Fenzl.		58[d]	63	57
Daucus guttatus Sibth. & Sm.		81[d]	86	84
Ducrosia anethifolia Boiss.		85[e]	84	84
Eryngium eburneum Decne.		93[d]	94	94
Oenanthe globulosa L.		99[e]	96	94
Petroselinum crispum (Mill.) Mansf.		93[e]	94	94

[a] Average of at least two analyses.

[b] Contains 9% 16:0 and 9% 18:2.

[c] Contains 13% 16:0 and 10% 18:2.

[d] TLC-UV method.

[e] TLC-GLC method.

Preparative TLC Methods

Used with GLC. TLC plates, 10x34 cm, were spread with 1-mm layers of diethyl-ether-washed Silica Gel G impregnated with 30% silver nitrate. Each plate was spotted with 25 (0.2 μl each) samples of Umbelliferae methyl esters. The chromatogram was developed with benzene in a 14x45-cm cylinder. Movement of the solvent front to 32 cm completely separated methyl petroselinate from methyl oleate. The bands containing the separated esters were scraped into individual flasks, and the adsorbent was extracted three times with ether. The solution was filtered, and the ether was removed under nitrogen. Ten milligrams of methyl myristate were weighed into a one-ml volumetric flask, and one of the recovered 18:1 fractions was quantitatively washed into this flask with ether. The flask was filled to the mark, and a 5-μl aliquot was analyzed by GLC in an F&M gas chromatograph, Model 810, equipped with a flame ionization detector and a 10 ftx1/8 in. copper column packed with 20% LAC-2-R 446. The other fraction of 18:1 was treated similarly, and the proportion of each was calculated by the following equations:

$$\frac{\text{Area 18:1 peak}}{\text{Area 14:0 peak}} \times \text{wt 14:0} = \text{wt 18:1}$$

$$\frac{\text{Wt } 18{:}1^{6} \times 100}{\text{Wt } 18{:}1^{6} + \text{wt } 18{:}1^{9}} = \%\ 18{:}1^{6} \text{ in } 18{:}1$$

Used with UV. TLC separation of methyl petroselinate and methyl oleate was carried out as for GLC except that the ether was distilled from sodium hydroxide pellets before extraction of the components. The esters were washed into individual one-ml volumetric flasks with spectral grade hexane and made to volume with this same solvent (no internal standard is used). These solutions were analyzed on a Beckman DK-2A recording spectrophotometer, which was continuously purged with dry nitrogen. The absorbance was determined at 182 mμ [molar absorptivity is 13,000 for both methyl petroselinate and oleate (10)], and the percentage of methyl petroselinate in the total 18:1 was calculated by the equation:

$$\frac{\text{Absorbance } 18{:}1^{6} \times 100}{\text{Absorbance } 18{:}1^{6} + \text{absorbance } 18{:}1^{9}} = \%\ \text{methyl petroselinate in total 18:1}$$

RESULTS AND DISCUSSION

The MRA procedure provided accurate analytical data on the five mixtures of known composition (Table I). Comparison of results from direct determination on Samples D and E, with data on the 18:1 fraction isolated by preparative GLC, indicates that the presence of methyl palmitate introduces no error, that the correction for methyl linoleate is entirely adequate, and that there is no fractionation of methyl oleate and methyl petroselinate in the preparative GLC.

In general, analyses of Umbelliferae esters either by direct MRA or by preparative GLC, followed by the MRA, gave results comparable with the TLC methods (Table I). Usually,

where results were not as close as desired, addition of preparative GLC helped to reconcile the percentages from the MRA and TLC methods. Since the esters were free of unsaponifiable material and since no unusual components were observed by GLC, the reason for the analytical differences is unknown. It was suspected that the 18:2 might include a component with Δ6 unsaturation which would make correction for methyl linoleate invalid; but isolation of the 18:2 component of *Daucus guttatus* by preparative GLC and location of its double bonds by MRA showed no Δ6 unsaturation present.

The MRA method is more straightforward and less time-consuming than the TLC method. The time required for the MRA method is about one-half hour whereas TLC plates require about three hours for the solvent to migrate 32 cm and another hour for the other steps in the method to be completed. Both methods are equally successful in determining the relative amounts of methyl petroselinate and methyl oleate.

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Reduction of Atherogenicity of Natural Fats by Small Additions of Ethyl Linoleate in the Diet of the Rat[1]

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ABSTRACT

Ethyl linoleate was substituted in part for the 20% of butterfat, hydrogenated coconut oil, lard, or tallow in an atherogenic diet fed to rats throughout a 40-week experimental period. Aortic degeneration, evidenced by lipid infiltration of the intima, was observed in the control groups but not in the linoleate-fed groups. Groups that received butterfat or hydrogenated coconut oil showed reduced plasma and hepatic cholesterol levels when fed 2% of ethyl linoleate; groups that received lard or tallow showed no significant change in plasma and hepatic cholesterol levels when fed 2% of ethyl linoleate; and groups that received a fat-free diet with 2% of ethyl linoleate showed lower plasma and hepatic cholesterol levels and more complete aortic protection than groups that were fed 20% of corn oil or cottonseed oil. The data suggest that, in the cholesterol-fed rat, the kind and amount of dietary fatty esters may influence aortic condition via some route(s) other than control of plasma and hepatic cholesterol levels.

INTRODUCTION

FROM THE TIME WHEN FATS of vegetable origin first were reported to have a hypocholesteremic action (1), research has been conducted in many laboratories to elucidate further the relationship of animal and vegetable fats to atherosclerosis. In most of the studies with purified fatty acids or their derivatives emphasis has been upon hypocholesteremic effects during periods which were too brief to permit observations on aortic or other vascular effects. Recent work in this laboratory has shown that 2% of purified ethyl linoleate can decrease plasma cholesterol in rats which have experimental hypercholesteremia and can give protection against formation of atheromatous plaques when fed to such rats in an otherwise atherogenic diet, which contains 20% of hydrogenated coconut oil as the only dietary fat (2).

It was the objective of the present work to investigate the effects of purified linoleate when added to other natural dietary fats which have been thought to be atherogenic; observations were to be made on aortic changes as well as on cholesterol levels of plasma and liver in the cholesterol-fed rat.

EXPERIMENTAL SECTION

Weanling rats (males, Wistar strain, 40-50 g), in individual wire cages, were fed ad libitum for 40 weeks a semipurified diet which contained (in percentage) casein, ether-extracted, 18; glucose (Cerelose), 55; salts No. 185, 4; cellulose (Cellu Flour), 2; cholesterol and sodium glycocholate (Nutritional Biochemical Corporation, Cleveland), 0.5 (each); fat 20; and a vitamin mixture, which was prepared as a premix with a portion of the casein. Details concerning sources and composition of the dietary items have been published elsewhere (3).

The fat, which was the variable in this diet, was supplied to the different groups as hydrogenated coconut oil (HCO), butterfat (Purdue Dairy Farm), lard, tallow, cottonseed oil, and corn oil (Mazola). Ethyl linoleate, when included, was prepared from safflower oil as a 98% pure, all-*cis* product (3,4) and was substituted for fat in the diet on an equal weight basis. One group of rats in each experiment was fed a fat-free diet, to which 2% of linoleate was added as the only lipid. This diet was adapted from that described above by increasing glucose to 77% and dividing the quantities of all other components by the factor 1.2. In all diets therefore the ratio of other nutrients to calories supplied as fat and carbohydrate was constant. Two complete experiments were performed at different times, with eight rats per group in Experiment I and 12 rats per group in Experiment II.

The fatty acid composition of the dietary fats (Table I) was determined by hydrolysis (5), esterification (6), and chromatography. For the chromatography, an F and M Model 500, programmed-temperature gas chromatograph was equipped with a 10-ft column of diethylene glycol adipate (DEGA, Gas Chrom P, Ap-

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TABLE I

Fatty Acid Distribution in the Dietary Fats as Determined by Gas-Liquid Chromatography

Dietary fat	Component fatty acid (%)							
	C <12	C 12	C 14	C 16	C 16:1	C 18	C 18:1	C 18:2
Hydrogenated coconut oil	28.3	39.2	16.2	7.2	....	7.3	1.8	....
Butterfat (W)[a]	10.1	3.2	8.5	37.4	3.6	11.5	23.6	2.1
Butterfat (S)[a]	22.6	5.4	13.2	24.5	2.5	9.8	19.1	1.9
Lard	....	1.1	2.1	28.8	4.5	12.2	39.8	11.5
Tallow	....	1.0	6.6	36.5	5.6	20.2	30.0	trace
Corn oil	....	....	....	14.1	....	2.6	29.3	54.0
Cottonseed oil	....	trace	trace	23.9	....	3.6	26.1	44.6

[a] W, winter butter; S, summer butter.

plied Science Laboratories, State College, Pa.). For quantitation under identical conditions, methyl ester standards, long-chained, were injected at 215C with 30.5 psi He (65-70 ml/min); short-chained, at 65C and a temperature increase of 4C/min to a maximum of 215C.

Blood samples were collected by heart puncture, from pentobarbital-anesthetized rats into a heparinized syringe, at 10-week intervals throughout the experiments. They were taken as quickly as possible after anesthetization and at the same time of day for each time period. After centrifugation the plasma was held in the frozen state until analyzed for cholesterol by the method of Sperry and Webb (7).

Livers and aortas were removed (to the level of the iliac artery) after 40 weeks of dietary treatment; livers were frozen until analyzed, and aortas were fixed in 10% formalin. Total hepatic lipid, extracted by the method of Harris and Gambal (8), was determined gravimetrically by evaporation of a portion of the extract. From the apical aortic arch and from the abdominal aorta just below the diaphragm about 25 frozen cross-sections (20 μ) were cut, stained with Oil Red O, and counterstained with alum hematoxylin. The degree of atherogenesis was estimated as percentage of the arterial surface area which showed lipid infiltration into endothelial or subintimal cells as evidenced by Oil Red O retention.

RESULTS AND DISCUSSION

Rats which consumed 20% of the more-saturated fats in their diets during the 40-week period showed extensive aortic damage (Table II). The damage was equally severe in both of the experiments and was generally more extensive in the abdominal aorta than in the aortic arch. Others (9,10) have generally found involvement of the thoracic aorta of the rat to be greater than that of the abdominal

TABLE II

Forty-Week Response to Partial Substitution of Ethyl Linoleate for Different Fats in an Atherogenic Diet; All Fats Were Fed at 20% of the Diet

Dietary fat	Ethyl linoleate substituted %	Body weight g		Plasma cholesterol mg/100 ml		Hepatic cholesterol mg/g wet wt.		Hepatic total lipid mg/g wet wt.		Aortic Lesions[a] Aortic arch %	Abdominal aorta %
		Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II		
HCO[b]	0	304	312	300	372	99	72	177	173	31	48
	2	531	631	151	140	47	32	129	126	0	0
	4	664	611	181	151	67	76	143	186	0	0
Butterfat	0	542	557	227	228	111	73	218	197	11	18
	2	582	588	163	131	59	52	159	147	0	0
	4	574	594	126	164	46	49	134	158	0	0
Lard	0	603	582	177	197	81	98	194	219	4	11
	2	595	576	226	169	78	76	185	190	0	0
Tallow	0	508	571	199	273	107	77	229	206	4	17
	2	536	624	232	214	71	81	136	183	0	0
Corn oil	0	648	546	140	140	80	72	213	180	<1	<1
CSO[b]	0	654	678	183	163	114	71	213	200	<1	<1
None	2	500	499	115	79	48	34	137	112	0	0

[a] % = Percentage of intimal surface involved in aortic lesions, estimated as follows: at least 25 sections each of apical aortic arch and abdominal aorta just below diaphragm were cut from each animal and graded visually as to surface area involved in lipid infiltration. Averages of these values for the two experiments are presented.

[b] HCO, hydrogenated coconut oil; CSO, cottonseed oil.

aorta, perhaps because of more drastic dietary treatment with high levels of cholesterol and bile salts and even the inclusion of thiouracil. Substitution of 2% or 4% of ethyl linoleate for a portion of the coconut oil, butterfat, tallow, or lard prevented gross lipid infiltration into endothelial and subintimal cells. The only arterial involvement in animals which received the linoleate was a small amount of stainable lipid observed in isolated aortic sections.

Although the lard contained sufficient 18:2 acid to provide more than 2% in the diet, a distinctly improved aortic condition was observed in both experiments when 2% of ethyl linoleate was substituted for an equal amount of lard in the diet. This suggests that linoleate as the simple ester may be more effective than an equal amount in the form of glyceride in a natural fat. Earlier studies (2) had shown that rats fed a diet low in fat derived arterial protection from dietary levels of ethyl linoleate which were no greater than those provided by the butterfat (Table I). However a higher fat level may increase the atherogenicity of the diet and tend to raise the linoleate level needed for a favorable response. The failure of the high-linoleate glycerides in corn oil and cottonseed oil to give arterial protection comparable with ethyl linoleate feeding suggests a need for further investigation of these relationships.

Although aortic lesions were generally associated with high cholesterol levels in plasma and liver, this relationship was not precise. The animals which were fed 2% of ethyl linoleate in the diet without any other dietary fat were free from aortic lesions and, of all groups, had the lowest cholesterol levels in their plasma and liver. On the other hand, small substitutions of ethyl linoleate for either lard or tallow gave aortic protection but produced only minor differences in plasma and liver cholesterol levels. Hepatic cholesterol levels were relatively high in the groups which were fed corn oil or cottonseed oil. It does not appear that the antiatherogenic effect of ethyl linoleate is exerted wholly through control of cholesterol in blood and liver.

Growth rates of the 20% HCO group without linoleate were retarded (15-20 g/wk vs. 25-30 g/wk for all other groups during the first 10 weeks), and these animals also showed other classical, essential fatty acid-deficiency symptoms including scaliness of tail, loss of hair, and increased water consumption. Survival rates of this group and the 2% linoleate (fat-free) group were relatively low (below 50% vs. > 66% for any other group). The minimal number finishing either experiment in any group was four rats. Some animals were lost during anesthetization and heart puncture.

Plasma cholesterol concentrations of rats fed 20% fat diets were essentially constant after the 10th week and were generally correlated with the degree of saturation of the dietary fat. Animals fed the 20% HCO diet had the highest concentration. Substitution of either 2% or 4% of ethyl linoleate for an equal amount of HCO produced plasma and liver cholesterol levels comparable with those of animals receiving 20% of either corn oil or cottonseed oil. Although similar results were obtained with substitutions in the 20% butterfat diet, linoleate substitution for lard or tallow produced no consistent lowering of plasma cholesterol.

A general effect of ethyl linoleate feeding was to lower hepatic cholesterol levels as well as plasma cholesterol levels. The ratio of hepatic cholesterol to plasma cholesterol was not altered in a consistent pattern by linoleate feeding, thus evidencing no redistribution effect between blood and liver during 40 weeks in this experimental model. This is consistent with the observations of Frantz and Carey (11), who found no evidence of a cholesterol shift from blood to liver in human beings as a result of corn oil feeding. In the liver, cholesterol constituted about one-third to one-half of the total lipid present, and this relationship was relatively consistent among the different groups irrespective of ethyl linoleate feeding.

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The Triglyceride Composition of 17 Seed Fats Rich in Octanoic, Decanoic, or Lauric Acid[1, 2]

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ABSTRACT

Seed fats of eight species of *Lauraceae* (laurel family), six species of *Cuphea* (Lythraceae family), and three species of *Ulmaceae* (elm family) were extracted, and the triglycerides were isolated by preparative thin-layer chromatography. GLC of the triglycerides on a silicone column resolved 10 to 18 peaks with a 22 to 58 carbon number range for each fat. These carbon number distributions yielded considerable information about triglyceride compositions of the fats.

The most interesting finding was with *Laurus nobilis* seed fat, which contained 58.4% lauric acid and 29.2-29.8% trilaurin. A maximum of 19.9% trilaurin would be predicted by a 1, 2, 3-random, a 1, 3-random-2-random, or a 1-random-2-random-3-random distribution of the lauric acid.[3] This indicates a specificity for the biosynthesis of a simple triglyceride by *Laurus nobilis* seed enzymes. *Cuphea lanceolata* seed fat also contained more simple triglyceride (tridecanoin) than would be predicted by the fatty acid distribution theories.

INTRODUCTION

THE DIRECT ANALYSIS of natural fat triglycerides by gas-liquid chromatography (GLC) was introduced in 1961-62 by Huebner (1) and Kuksis and McCarthy (2). It has proven to be a rapid and quantitatively accurate method for separating triglyceride mixtures by molecular weight. Two recent reviews (3, 4) have discussed the operating procedures and applications of this technique.

Triglyceride GLC is most useful for the analysis of fats containing a wide range of fatty acid molecular weights and hence a wide range of triglyceride molecular weights. The authors have already shown how fats containing C_{20}, C_{22}, and C_{24} fatty acids can be effectively analyzed by this method (5). The present work was undertaken to demonstrate how useful GLC can be for analysis of natural fat triglycerides containing C_8, C_{10}, and C_{12} acids. Seventeen seed fats rich in octanoic, decanoic, or lauric acid have been analyzed for triglyceride composition by using GLC.

EXPERIMENTAL

Materials

Actinodaphne hookeri seeds were obtained through the courtesy of N. L. Murty, Bombay, India. The seeds were collected in June 1962 from trees in the Bhandardara Hills, Ahmednagar District, Maharastra, India. Other seeds were purchased from the following suppliers: *Cinnamomum camphora* and *Lindera benzoin* from Herbst Brothers Seedsmen Inc., Brewster, N. Y.; *Laurus nobilis, Cuphea lanceolata,* and *Cuphea llavia* from Thompson and Morgan Ltd., Ipswich, England; *Lindera praecox* and *Lindera triloba* from Harry E. Saier, Dimondale, Mich.; *Sassafras albidum* and *Ulmus pumila* from Forestry Associates, Allentown, Pa.; *Umbellularia californica* from Western Seeds, Agness, Ore.; *Cuphea ignea* from Germania Seed Company, Chicago, Ill.; *Ulmus americana* and *Zelkova serrata* from F. W. Schumacher, Sandwich, Mass.

Samples of *Cuphea carthagenensis, Cuphea hookeriana,* and *Cuphea painteri* seed fats were provided by F. R. Earle, Northern Regional Research Laboratory, Peoria, Ill.

Methods

Gas-Liquid Chromatography of Triglycerides. GLC analysis of triglycerides was achieved by using the general methods of Litchfield, Harlow, and Reiser (4). The gas chromatograph (F&M 400) was equipped with a hydrogen flame detector and automatic temperature programming. The 0.53 m x 2.5 mm I.D. glass or stainless steel column contained 3.0% JXR silicone on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.) and was programmed from 170C to 340C at 4C/min with a helium gas flow of 100 ml/min. The peaks were identified as to carbon number by co-chromatography with known compounds. Quantitation procedures were the same as previously described (4). Results are reported in mole percentage.

Gas-Liquid Chromatography of Fatty Acids.

[1] Presented at the AOCS Meeting in Los Angeles, April 1966.

[2] This paper is taken from a thesis to be submitted to Texas A&M University by R. D. Harlow in partial fulfillment of the requirements for a Master of Science degree.

TABLE I
Fatty Acid Composition (mole %)

	Oil content	Component analyzed[a]	Fatty acids														References to previous work
			8:0	10:0	10:1	12:0	12:1	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	20:1	
Lauraceae	%																
Actinodaphne hookeri	73.5	S–SC	—	2.2	—	93.4	—	2.2	—	0.4	—	tr	1.6	0.2	—	—	16,17
Cinnamomum camphora	65.2	S–SC	0.5	63.0	—	34.5	—	0.7	—	0.1	—	tr	1.0	0.2	tr	tr	9,18
Laurus nobilis	29.4	S–SC	—	1.5	—	58.4	—	1.6	—	4.4	—	0.4	17.7	15.0	0.2	0.8	9,11,19 20,21,22
Lindera benzoin	35.6	S–SC	0.2	47.0	—	45.3	—	2.1	—	0.4	—	0.1	2.4	2.3	0.1	0.1	9
Lindera praecox	32.7	S–SC	0.5	59.4	—	27.4	0.5	3.8	0.1	0.9	0.1	0.2	5.4	1.5	0.1	0.1	9
Lindera triloba	63.9	S–SC	tr	6.1	4.3	35.3	37.7	3.9	5.2	0.3	0.1	0.2	5.1	1.7	—	0.1	—
Sassafras albidum	52.5	S–SC	7.5	69.3	—	19.8	—	0.6	—	0.2	—	tr	2.2	0.3	0.1	tr	10
Umbellularia californica	43.3	S–SC	0.5	28.1	—	66.7	—	1.6	—	0.2	—	0.2	1.9	0.6	0.1	0.1	9
Lythraceae																	
Cuphea carthagenensis	—	—	5.3	20.6	—	55.6	—	8.0	—	2.1	—	0.5	3.6	4.1	0.1	0.1	12
Cuphea hookeriana	—	—	71.5	23.9	—	0.1	—	tr	—	0.8	—	0.2	1.0	2.3	0.2	tr	12
Cuphea ignea	41.7	S	4.5	90.3	—	0.6	—	0.1	—	1.2	—	0.1	1.1	2.0	0.1	tr	12
Cuphea lanceolata	21.9	S	0.7	86.6	—	2.0	—	1.6	—	2.4	—	0.4	2.1	3.6	0.5	0.1	—
Cuphea llavia	23.7	S	1.0	91.4	—	1.1	—	0.6	—	1.2	—	0.2	2.2	2.1	0.2	0.1	7,10,12
Cuphea painteri	—	—	77.8	19.6	—	0.1	—	tr	—	0.6	—	0.1	0.6	1.1	0.1	—	12
Ulmaceae																	
Ulmus americana	29.6	S–SC	9.6	69.7	—	3.6	—	1.8	—	3.9	—	0.9	5.4	4.3	0.7	0.1	15
Ulmus pumila	28.4	S+P	8.9	64.9	—	3.8	—	3.3	—	6.0	—	0.7	5.2	5.4	1.7	0.1	14
Zelkova serrata	47.4	S–SC	11.5	77.9	—	3.2	—	0.6	—	1.7	—	0.3	2.4	2.0	0.3	0.1	10,13

[a] S = seed, S–SC = seed minus seed coat, S+P = seed plus part of pericarp.

TABLE II
Triglyceride Composition (mole %)

	Carbon number																		Average fatty acid chain-length	
	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	From triglyceride GLC data	From methyl ester GLC data
Lauraceae																					
Actinodaphne hookeri	—	—	—	—	—	0.2	6.1	85.4	4.9	0.3	1.3	0.4	0.3	0.9	0.1	0.1	tr	—	—	12.09	12.12
Cinnamomum camphora	—	—	—	0.2	11.4	68.5	15.2	1.2	1.0	0.8	0.2	0.2	1.1	0.2	—	—	—	—	—	10.82	10.81
Laurus nobilis	—	—	—	—	—	0.2	3.5	29.8	1.1	1.4	16.3	2.8	6.8	21.5	3.2	4.3	8.1	0.9	0.1	14.42	14.24
Lindera benzoin	—	—	—	0.1	8.4	36.0	38.9	7.0	1.9	2.5	1.5	0.6	1.3	1.6	0.1	tr	0.1	—	—	11.30	11.41
Lindera praecox	—	—	—	0.5	17.4	35.3	22.5	6.3	7.5	4.2	1.0	0.8	2.8	1.4	0.1	0.1	0.1	—	—	11.33	11.35
Lindera triloba	—	—	—	—	—	2.2	19.2	47.8	15.7	2.8	4.2	2.3	1.0	4.2	0.6	—	—	—	—	12.36	12.42
Sassafras albidum	—	—	0.3	9.3	43.5	31.0	8.1	1.6	2.9	1.6	0.3	0.1	0.9	0.4	—	—	—	—	—	10.50	10.49
Umbellularia californica	—	—	—	—	1.1	11.9	61.9	19.7	1.3	1.0	1.2	0.3	0.4	1.1	0.1	—	—	—	—	11.52	11.63
Lythraceae																					
Cuphea carthagenensis	—	—	—	0.3	3.1	14.7	31.7	24.6	8.6	3.5	2.3	1.9	2.8	3.1	0.8	0.9	1.5	0.2	—	12.13	12.13
Cuphea hookeriana	0.3	40.4	40.6	11.8	0.3	0.7	1.6	0.8	0.1	tr	0.2	0.4	0.2	tr	0.1	0.8	1.7	tr	—	8.85	8.92
Cuphea ignea	—	—	0.3	11.4	78.6	1.5	0.7	2.5	2.8	0.1	tr	0.3	0.6	tr	tr	0.4	0.8	—	—	10.21	10.26
Cuphea lanceolata	—	—	—	2.2	70.0	5.4	4.0	5.2	9.3	0.8	0.3	0.4	0.8	0.1	0.2	0.4	0.9	—	—	10.65	10.77
Cuphea llavia	—	—	—	2.3	79.0	3.0	1.5	3.2	9.3	0.4	0.1	0.2	0.5	tr	tr	0.1	0.4	—	—	10.43	10.49
Cuphea painteri	0.3	49.9	37.8	7.8	0.2	0.5	0.8	0.3	tr	tr	0.2	0.4	0.2	tr	0.2	0.6	0.8	tr	—	8.62	8.63
Ulmaceae																					
Ulmus americana	—	—	1.2	15.6	41.1	7.1	5.0	9.3	11.3	1.7	1.3	2.3	3.1	0.4	0.2	0.2	0.2	—	—	10.93	11.10
Ulmus pumila	—	—	0.9	12.8	34.2	7.2	7.3	11.8	12.1	2.1	2.2	3.3	3.6	0.5	0.5	0.8	0.7	—	—	11.29	11.44
Zelkova serrata	—	0.1	2.5	24.7	52.9	5.5	2.2	4.3	4.8	0.6	0.3	0.7	1.2	0.1	tr	0.1	tr	—	—	10.22	10.37

Fatty acid composition was determined by GLC analysis of the methyl esters, prepared by heating the triglycerides for 60 min at 100C with 5% HCl/CH_3OH in a sealed vial (6). After cooling and the addition of water the methyl esters were extracted in 1–2 ml of petroleum ether and injected directly into the gas chromatograph. To avoid any loss of low-molecular-weight esters, no solvent was evaporated after extraction. The methyl esters were analyzed on a 1.82 m x 2.4 mm I.D. column containing 20% diethylene glycol succinate polyester coated on 60/80 mesh Chromosorb W, as previously described (7). All fatty acid compositions are reported in mole percentage.

Procedure. Each seed sample was sorted to remove damaged seeds and foreign material. Where practical, the seed coat was removed from the endosperm (Table I). The seeds were then ground in a Waring Blendor, weighed into a tared paper thimble, and extracted with petroleum ether (30-60C bp) for 4 hr in a Soxhlet extraction apparatus. The oil content (wet basis) of each seed is reported in Table I.

The triglycerides were isolated from each seed fat by using preparative thin-layer chromatography on 1.0-mm thick plates of silicic acid impregnated with Rhodamine 6G. Plates were developed in a 79/20/1 mixture of petroleum ether/diethyl ether/acetic acid. The triglyceride band was located under ultraviolet light, scraped into a beaker, placed in a small chromatography column, and eluted with diethyl ether. Visual examination of the chromatoplates indicated that the triglyceride constituted more than 85% of the total fat in all cases.

RESULTS

Each purified triglyceride sample was analyzed for fatty acid and triglyceride composition by GLC. Fig. 1 illustrates the peak resolution obtained in the triglyceride chromatograms. Quantitative results are reported in Tables I and II. The average fatty acid chain-length was calculated from the triglyceride composition of each fat, and also from the methyl ester GLC data by using the method of Kuksis et al. (8). Comparison of the two values (Table II) showed close agreement for all 17 fats, indicating that the carbon number distributions reported in Table II are close to the correct values.

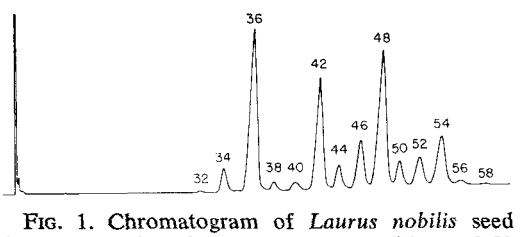

FIG. 1. Chromatogram of *Laurus nobilis* seed fat triglycerides. GLC operating conditions: 0.53 m x 2.5 mm I.D. glass column, packed with 3.0% JXR on 100/120 mesh Gas-Chrom Q; 100 ml/min. He carrier gas; column temperature programmed 170 →340C at 4C/min.

DISCUSSION

Fatty Acid Composition

All but two of the 17 seed fats analyzed have been examined for fatty acid composition by previous workers. References to these other investigations are listed in Table I. With the exception of *Sassafras albidum* the fatty acid compositions found were in general agreement with the literature although in some cases the ratio of the major component acids was different. This is not an uncommon phenomenon in seeds of the same species which come from different sources (23).

The authors were unable to detect any hexanoic acid in *Sassafras albidum* triglycerides although Earle et al. (10) reported finding 13% 6:0 in the total seed fat. The methyl ester preparation procedure was designed to avoid evaporation of volatile esters so that it seems reasonable some hexanoic acid would have been detected had it been present. The GLC of *Sassafras albidum* triglycerides showed no appreciable amount of triglycerides below carbon number 28, which would also indicate the virtual absence of 6:0 in a fat containing 69.3% 10:0. Perhaps the hexanoic acid reported by Earle et al. (10) is of nontriglyceride origin or is some other substance which elutes at the same time as 6:0 on GLC.

The complete fatty acid composition of *Lindera triloba* seed fat is reported for the first time. This unusual fat contains 35.3% lauric acid and 37.7% dodecenoic acid as well as small amounts of decenoic and tetradecenoic acids. The monoene character of the 10:1, 12:1, and 14:1 esters was established by separating *Lindera triloba* methyl esters by Ag^+ thin-layer chromatography (24), recovering the monoene band, and analyzing the monoene methyl esters separately by GLC. The chain lengths of the 10:1, 12:1, and 14:1 were confirmed by GLC on a 2-ft JXR dimethylpolysiloxane column. These acids are probably the *cis*-4-decenoic, *cis*-4-dodecenoic, and *cis*-4-tetradecenoic acids which Hopkins et al. (9) found in *Lindera umbellata* seed fat. It is interesting to note that not all *Lindera* species contain these unusual acids.

The fatty acid composition of *Cuphea lanceolata* seed fat is also reported for the first time. It closely resembles the composition of *Cuphea llavia* seed fat.

Triglyceride Composition

The triglyceride compositions of the 17 seed fats investigated are listed in Table II. From 10 to 18 peaks within a 22 to 58 carbon number range are resolved for each fat. The GLC of the triglycerides is rapid and quantitative. The chromatogram is available 45 min after sample injection, and quantitation takes 2 hr by triangulation or 30 min with a mechanical integrator. The carbon number distributions which were found yielded considerable information about the triglyceride composition of a fat when a wide range of fatty acid molecular weights are present. Even more detailed information can be gained if the triglyceride mixtures are fractionated by Ag^+ chromatography (7) or liquid-liquid partition chromatography (25) prior to GLC analysis.

All the seed fats examined show a complex pattern of triglyceride composition. This would, of course, be expected with the wide range of fatty acids in each fat. However the main triglyceride peaks always correspond to the triglycerides containing only the major fatty acids. For example, *Zelkova serrata* contains 77.9% 10:0, and its major triglyceride peak is C_{30}, corresponding to tridecanoin. *Actinodaphne hookeri* (16) and *Cuphea llavia* (7) triglycerides have been examined previously. Results confirm these earlier findings but present a more detailed analysis. The triglyceride compositions of two fats, *Laurus nobilis* and *Cuphea lanceolata,* show some unusual features and will be discussed in detail below.

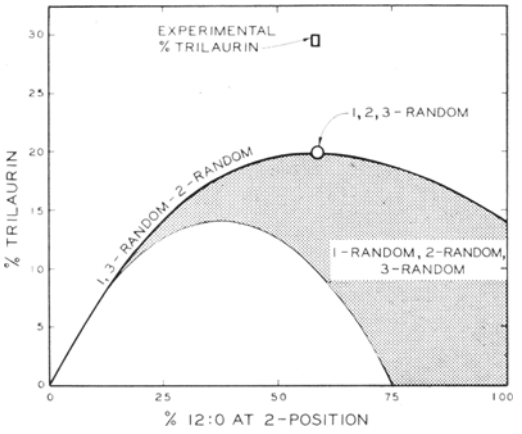

FIG. 2. Possible range of trilaurin content in *Laurus nobilis* seed fat as predicted by the 1,2,3-random (single point), the 1,3-random-2-random (heavy line), and the 1-random-2-random-3-random (shaded area) distribution hypotheses.

Laurus nobilis. Collin and Hilditch (19, 21) and Bömer and Ebach (26) have reported that *Laurus nobilis* seed fat contains an exceptionally high amount of trilaurin. The *L. nobilis* fat which was examined contained 58.4% lauric acid and 29.8% C_{36} triglyceride. The amount of trilaurin in this C_{36} peak can be estimated by the following indirect calculation. *L. nobilis* seed fat contains only one fatty acid with a lower molecular weight than lauric acid; that is the 1.5% 10:0. Therefore the C_{32} and C_{34} GLC peaks must contain some decanoic acid. The C_{32} peak contains only one possible triglyceride: $C_{10}C_{10}C_{12}$. Since the C_{32} peak represents 0.2% of the total triglycerides, then $\frac{2}{3} \times 0.2 = 0.13\%$ 10:0 must be in the C_{32} peak. The C_{34} peak contains only two possible triglycerides: $C_{10}C_{12}C_{12}$ and $C_{14}C_{10}C_{14}$. The C_{34} peak would contain the minimum amount of decanoic acid if the peak contained only $C_{10}C_{12}C_{12}$. The C_{34} peak represents 3.5% of the total triglycerides so at least $\frac{1}{3} \times 3.5 = 1.17\%$ 10:0 must be in the C_{34} peak. Since *L. nobilis* seed fat contains only 1.5% decanoic acid, and a minimum of $0.13 + 1.17 = 1.30\%$ of this must be in the C_{32} and C_{34} peaks, then the maximum amount of 10:0 that could possibly occur in the C_{36} peak would be $1.50 - 1.30 = 0.2\%$ 10:0. Now the C_{36} peak contains only three possible triglycerides: $C_{12}C_{12}C_{12}$, $C_{10}C_{12}C_{14}$, and $C_{10}C_{10}C_{16}$. The maximum non-trilaurin content of the C_{36} peak would occur if all the remaining 0.2% 10:0 were in the form of $C_{10}C_{12}C_{14}$. Under such conditions the C_{36} peak would consist of $3 \times 0.2 = 0.6\%$ $C_{10}C_{12}C_{14}$. and $29.8 - 0.6 = 29.2$ trilaurin. Thus the minimum trilaurin content of *L. nobilis* seed fat is 29.2%. Of course the maximum amount of possible trilaurin would be 29.8%, the total C_{36} material present. Therefore the above calculations show that the trilaurin content of *L. nobilis* seed fat must lie between 29.2 and 29.8%. This agrees fairly closely with the 30 weight % trilaurin found by Bömer and Ebach (26) and the 35.1 mole % trilaurin reported by Collin (21) with crystallization studies.

The presence of 29.2-29.8% trilaurin in a seed fat containing only 58.4% lauric acid is quite unusual, and this has been commented upon by Collin and Hilditch (19, 21). Therefore it is interesting to compare the trilaurin content of *L. nobilis* seed fat with that predicted by the various fatty acid distribution hypotheses. This is done graphically in Fig. 2

by plotting % trilaurin *versus* % 12:0 at the 2-position of the total triglycerides. The % 12:0 at the 2-position is varied from 0-100% to cover all possible cases. The 1, 2, 3-random distribution theory[3] predicts 19.9% trilaurin, which is represented as a point in the center of the graph. The 1, 3-random-2-random distribution theory (27, 28) predicts the amount shown by the heavy line at the top of the shaded area. And the 1-random-2-random-3-random distribution theory (29, 30) predicts the amount of trilaurin shown by the shaded area. The interesting point is that none of these three distribution hypotheses can possibly account for more than 19.9% trilaurin regardless of the distribution of lauric acid on the three positions of the glycerol molecule. Since 29.2-29.8% trilaurin is present, it is obvious that none of the three distribution hypotheses tested can correctly predict the triglyceride composition of *L. nobilis* seed fat.

Experimental results indicate a distinct specificity for the biosynthesis of a simple mono-acid triglyceride by *L. nobilis* seed enzymes. It is easy to speculate on possible causes for this tendency: a) only lauric acid is available for triglyceride biosynthesis during one stage of seed development (21); b) one region of the seed is much richer in lauric acid than the other parts; c) *L. nobilis* seed contain more than one pathway for the biosynthesis of triglycerides; or d) the enzyme specificities for triglyceride biosynthesis in this seed are considerably different from other seeds. For the present however, such explanations are pure speculation; and further study is needed to explain properly the unusually high percentage of trilaurin in *L. nobilis* seed fat.

Cuphea lanceolata. This seed fat is another interesting example of fat containing more than the expected amount of a simple triglyceride. *Cuphea lanceolata* seed fat contains 86.6% 10:0 with only one lower-molecular-weight fatty acid, 0.7% 8:0. By examining the triglyceride compositions of the C_{28} and C_{30} peaks in the manner described above, one can estimate the tridecanoin content of this fat. The C_{28} peak contains only one possibly triglyceride: $C_8C_{10}C_{10}$. Since the C_{28} peak represents 2.2% of the total triglycerides, it must contain 2.2/3 = 0.73% of 8:0. But the total fat contains only 0.7% 8:0 so all of this is accounted for in the C_{28} peak. Therefore the C_{30} peak contains no 8:0, and the C_{30} peak must be all tridecanoin. *Cuphea lanceolata* seed fat contains 70.0% tridecanoin. In a graph similar to Fig. 2, a maximum of 64.9% tridecanoin would be predicted by a 1, 2, 3-random, a 1, 3-random-2-random, or a 1-random-2-random-3-random distribution of the decanoic acid. Thus *Cuphea lanceolata* fat does not follow any of the above distributions theories, and its seed enzymes show a definite specificity for the biosynthesis of a simple mono-acid triglyceride.

[3]The confusing nomenclature of fatty acid distribution hypotheses using "random" in their titles has been simplified by adopting three easily understood terms. In a *1,2,3-random pattern*, one pool of fatty acids is randomly distributed to all three positions of the glycerol molecule. In a *1,3-random-2-random pattern*, two pools of fatty acids are randomly and separately distributed to the 1,3- and 2-positions of the glycerol molecule. In a *1-random-2-random-3-random* pattern, three pools of fatty acids are randomly and separately distributed to the 1-, 2-, and 3-positions of the glycerol molecule.

ACKNOWLEDGMENT

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SHORT COMMUNICATION

An Autosampler for Solvent-free Sample Introduction into a Gas Chromatograph

THE HIGH SENSITIVITY and precision obtained in gas chromatography has made it a tool of the utmost importance in lipid analysis. The manual sample introduction is however a serious limitation in effective routine analysis of a large number of samples.

During the analysis of many similar samples of fatty acid methyl esters from rapeseed obtained in plant-breeding projects, the need arose for a completely automatic GLC-system. Being unable to obtain such a system commercially, we constructed and assembled one at our laboratory. A preliminary report on the system was given in 1964 (1). The essential part of the new construction, viz., the automatic sample inlet is presented here. A detailed description of the whole system as well as the types of compounds to be successfully analyzed will be dealt with in forthcoming reports.

Figure 1 shows a cutaway drawing and Figure 2 an actual photograph of the sample inlet system, the main parts of which are the sample storage unit and the vaporizer unit (Figures within brackets refer to the numbers on Figs. 1 and 2.) The former contains a rotor [1] made of light metal with 60 chambers [2] 2x2 mm wide and 17 mm deep. The rotor fits into the rotor housing [5], over which a round Perspex screen is placed. A rubber O-ring and 6 screws [7] provide good frictional contact between the Perspex and the rotor housing.

The vaporizer consists of a stainless steel unit [16] with carrier gas inlet [19] and outlet [20], to which the separation column is fitted. The upper part of the stainless steel unit is connected to the rotor housing through an airtight stainless steel fitting [25], which is tightened to the lower unit through a silicon rubber packing and to the upper unit through a cone which is pressed to the socket in the rotor housing via a system of metal sheets and clamps [26, 27]. The clamps are screwed to the metal stands [15] of the rotor housing.

In the central part of the vaporizer tube a piston [17] is held in position by a spring [18]. Through the piston a hole is drilled so that the carrier gas can pass. To the lower part of the piston tube a metal tube [29] is connected, which is fitted to the carrier gas line via an electric valve (normally open) [31] and to the room atmosphere through another valve, normally closed. To the left side of the

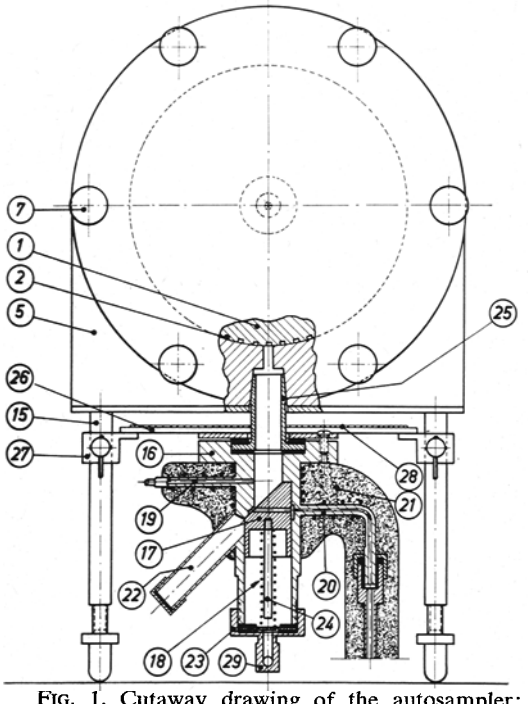

FIG. 1. Cutaway drawing of the autosampler: 1 = rotor, 2 = storage chamber, 17 = piston, 18 = spring, 19 = carrier gas inlet, 20 = carrier gas outlet, 22 = trap for empty sample tubes.

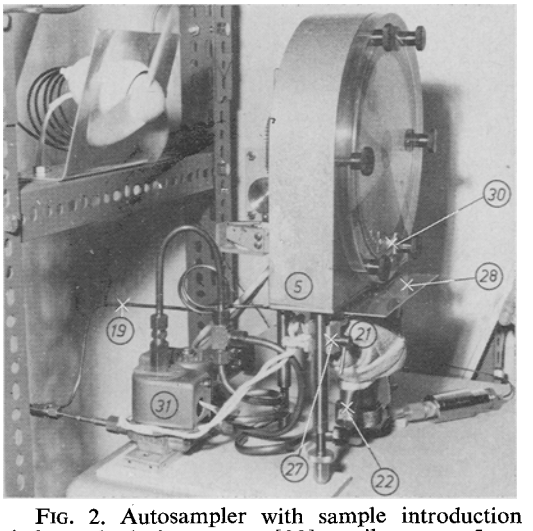

FIG. 2. Autosampler with sample introduction hole and closing screw [30] easily seen: 5 = rotor housing, 19 = carrier gas line, 21 = vaporizer, 22 = trap for empty sample tubes, 28 = heat reflector, 31 = gas valve.

vaporizer unit a side tube [22] is fitted. The vaporizer unit is heated by heating tape to 275C and insulated [21]. The rotor is kept at room temperature by a fan playing from behind and by reflecting the heat from the vaporizer with a polished aluminum sheet [28].

Fatty acid methyl esters are deposited from a chloroform solution on the inside of small metal tubes (about 2 mm O.D. and 1 mm I.D., 5-mm long), open at both ends. These tubes are introduced one at a time through a hole later closed by a screw [30] in the Perspex sheet (Fig. 2) into all the compartments but the one over the opening to the vaporizer by revolving the rotor clockwise with a manual control-unit. During this process argon is flushed through the sample introduction hole into all sample chambers as they are filled.

The rotor is rotated clockwise one step (controlled by a timer), and the small metal tube drops on the piston [17] where the sample rapidly vaporizes. The argon carrier gas then transports the vaporized sample on to the column. At a suitable time the timer unit causes a decrease in the gas pressure, which is about 1.4-1.6 atm in both the rotor and carrier gas line, to atmospheric pressure in the space under the piston by closing the open and opening the closed valve. This makes the piston move downward. After one second the valves are returned to previous positions, and the piston moves upward under the influence of the spring [18]. In the meantime the empty sample tube falls into the side container [22].

The unit has been found to function properly for methyl esters of oils from rape and similar seeds as such esters were not changed to a measurable extent when stored solvent-free in argon atmosphere for 24 hr or more. A 40-cm-long column packed with 10% silicon oil on Celite 60/80 mesh and a ^{90}Sr ionization detector have been used in our system, which also incorporates a digital recording of peak areas. The data received in 15 consecutive analyses of a single sample of methyl esters from turnip rapeseed, containing 18:3 as the most unsaturated ester, are shown below.

Mean and standard deviation	C_{16}	C_{18}
	2.3 ± 0.08	48.1 ± 0.27
C_{20}	C_{22}	
10.5 ± 0.38	39.1 ± 0.48	

It is obvious that the precision is good and completely satisfactory for routine analysis. Our automatic GLC has been successfully used for similar samples during a period of 4 years.

After the completion of our machine Podmore (2), Tinti (3), and Ruchelman (4) presented dry samplers primarily to be used for steroid separations.

Compared with the last two of these autosamplers, this construction offers the advantage of withdrawing the empty sample container, thus preventing a large number of sample tubes from accumulating on top of the column and making a frequent disassembly of the unit unnecessary. The presence of such tubes may cause a variable band broadening of the vaporized sample. Furthermore any high boiling compounds not immediately vaporized may slowly decompose, thereby causing detector irregularities.

The rotor in the unit described by Tinti is continuously moving whereas our rotor turns exactly 1/60 of a full revolution to introduce a new sample. The whole rotor in our machine can be loaded at one time compared with only half of the former as our sample compartments are parallel to the rotor axis and not perpendicular.

Ruchelman placed wire gauzes in Teflon cups, on which to deposit the sample from a large solvent volume. The part of the sample deposited on the outside of the wire gauze might cause contamination of the storage cabinet. This assumption is based on our experiences at an early stage when part of our samples, although deposited on the inside of the metal capillaries, spread to the outside because of surface tension. An impregnation of the inside of the metal capillaries with a silicon oil, followed by heating to 300C for some hours, solved this problem.

The system devised by Podmore (2) utilizes impregnated metal cylinders, which are withdrawn from the vaporizer zone after a short exposure. The way of arranging the samples in the storage tube is however critical, and the sample capacity appears to be rather limited.

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Excretion of Lipids by the Liver Fluke (*Fasciola Hepatica* L)

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ABSTRACT

Adult liver flukes kept in a glucose-enriched medium were found to excrete lipids. Analysis of the incubation medium showed that both neutral lipids (including cholesterol and its esters) and polar lipids were released. The rate of lipid excretion was greatly reduced when the excretory pores and mouths of the flukes were ligated. Histochemical examination of the flukes indicated that such lipids, released through the excretory pores, originate in the cells lining the excretory ducts.

INTRODUCTION

THE OBSERVATION IN THIS LABORATORY of an iridescent film on the surface of media in which adult flukes were being incubated suggested the presence of excreted lipids. Several workers (1-3) have demonstrated histochemically the presence of lipids in the region of the excretory system of the adult liver fluke and the passage of fat droplets through the excretory pores. Further examination of the excreted globules failed to reveal the presence of cholesterol and its esters or "lipins" although some unsaturated lipid was indicated by osmic acid treatment (1). The absence of cholesterol was based on negative results with rather insensitive histochemical techniques, and tests for the so-called "lipin" fraction, presumed to be mainly phosphatidyl ethanolamine and phosphatidyl serine, were inconclusive. Failure to demonstrate cholesterol histochemically in the fat droplets of the excretory vessels led von Brand (4) to the conclusion that no simple excretion of exogenous food lipid is involved and that the excreted fat must therefore originate in the body of the worm. More recent work by Clegg and Morgan (5) however provided evidence that cholesterol and its esters were present in the neutral lipid fraction of extracts of the posterior portions of adult flukes.

A more detailed investigation of lipid excretion from the liver fluke is clearly desirable, and the present communication reports the preliminary findings of such a study.

EXPERIMENTAL SECTION

Liver flukes were collected from the bile ducts of freshly killed cattle and immediately transferred to an incubation medium for transport at ambient temperature to the laboratory, where they were transferred to fresh medium. The medium used throughout this investigation was a solution essentially as described by Dawes (6). The composition was as follows: NaCl, 3.8 mg/ml; KCl, 0.3 mg/ml; $CaCl_2$, 0.1 mg/ml; Na_2HPO_4, 0.5 mg/ml; and $MgSO_4$, 0.3 mg/ml. To reproduce more nearly the conditions in hepatic bile, $NaHCO_3$, 4.7 mg/ml, was added, giving HCO_3- and Cl- concentrations of 35.3 and 81.1 meq/L respectively. Penicillin 500 U/ml and streptomycin sulphate 0.5 mg/ml (Crystamycin, Glaxo Laboratories) were also added. For some experiments the medium was enriched with glucose (1 mg/ml).

Lipid analyses of the glucose-containing medium (2-5 ml/fluke) were made after 4 hr of incubation, and histochemical examinations were made on flukes which had been incubated either with or without glucose for 12 hr. All incubations were carried out at 37C. In a few experiments flukes were kept in the glucose-enriched medium for 24 hr after mouth openings and excretory pores had been closed by ligating with sterilized cotton thread.

Lipids were extracted from the medium by the addition of chloroform and methanol to give a final ratio by volume of $CHCl_3$: MeOH:H_2O (2:2:1.8) (7). After mixing with a Silverson vortex homogenizer, the resulting mixture was centrifuged for 10 min at 1700 *g* to give clear chloroform and methanol phases with an interfacial skin. The upper methanol phase was aspirated and discarded before transferring the chloroform phase to a tared, round-bottomed flask fitted with a ground glass joint, leaving the interfacial skin in the tube. Solvent was then removed on a rotary evaporator at 37C under reduced pressure. A few extractions were made by the method of Folch, Lees, and Stanley (8), but

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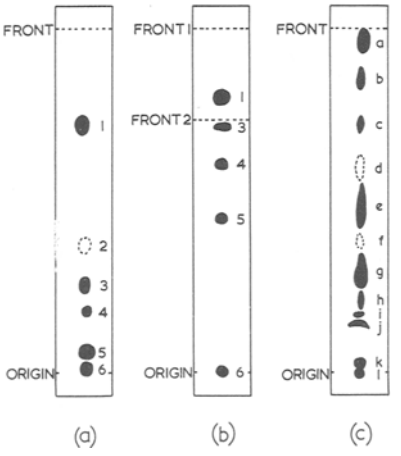

FIG. 1. Thin-layer chromatograms of excreted fluke lipids. Minor components are shown in dotted outline.

(a) and (b) show separation of total lipids in solvent systems 1 and 2 respectively. Spots were identified as 1, cholesterol ester; 2, unknown, possibly glycerylether diester; 3, triglyceride; 4, free fatty acid; 5, cholesterol; 6, polar lipid.

(c) shows separation of total lipids in solvent system 3. Some of the spots were tentatively identified as a, neutral lipid; b, phosphatidyl ethanolamine; d, phosphatidyl serine (phosphatidyl inositol?); e, phosphatidyl choline; g, sphingomyelin; h, lysolecithin?

no chromatographic differences were seen in lipid fractions obtained by the two methods. The resulting total lipid fraction was dried over anhydrous calcium sulphate in a vacuum desiccator, weighed, and redissolved in a small volume of heptane or chloroform for subsequent analysis by thin-layer chromatography (TLC). Lipids were separated on 200 μ layers of Silica Gel G (Camag), pre-activated by heating for 30 min at 100-110C, and cooled in a vacuum desiccator. The following solvent systems were used.

For Neutral Lipids

Solvent System 1. Hexane or petroleum ether (b.p. 60C-80C)-diethyl ether-acetic acid (90:10:1) (9).

Solvent System 2. Petroleum ether (b.p. 60C-80C)-diethyl ether-acetic acid (70:30:1) run to 15 cm, followed, after drying the plate, by diethyl ether-petroleum ether (b.p. 60C-80C)-acetic acid (70:30:1) run to 11 cm, in the same dimension (10).

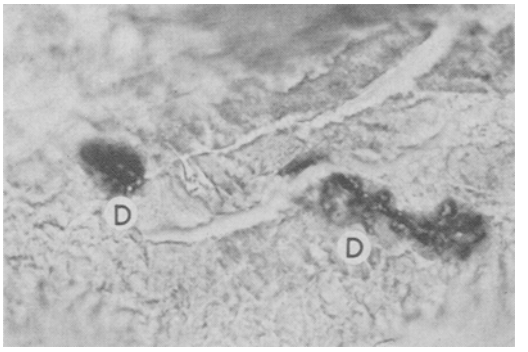

FIG. 2. Section of liver fluke stained with Oil Red O (x 1150.) Fluke incubated for 12 hr in glucose-enriched medium. Excretory ducts (D) contain neutral lipids in both lining cells and lumina.

For Polar Lipids

Solvent System 3. Chloroform-methanol-acetic acid-water (65:15:10:4) (11).

Spots were visualized either by iodine vapor or by spraying the plates with 50% aqueous sulphuric acid, followed by slow charring in an oven at 200C.

Flukes for histochemical examination were fixed for 24 hr in 4% formaldehyde saturated with calcium chloride (12), washed in tap water, and impregnated for 4 hr in two changes of Aquax (G. T. Gurr Ltd., London) at 50C. Transverse sections were cut at 3 μ and submitted to one of the following procedures: the Oil Red O method for neutral lipids (13), the copper phthalocyanin method for phospholipids (14), Fischler's method for fatty acids (15), the digitonin reaction for cholesterol (16), and the Okamoto method for cholesterol and its esters (15).

RESULTS

Lipid Analysis of Incubation Media

Extraction of lipids from glucose-enriched medium showed, in four separate experiments involving a total of 6.5 g fluke, that the rate of total lipid excretion was in the range of 0.5 to 1.2 mg/g fluke/hr. In three further experiments, in which 3.0 g of flukes with ligated oral and excretory openings were incubated for 24 hr, lipid release into the medium was reduced to less than 0.1 mg/g fluke/hr.

Separation of total lipids by TLC in solvent systems 1 and 2 is illustrated in Fig. 1. Comparison with standards showed the presence in the neutral lipid fraction of both free cholesterol and its long-chain fatty acid esters (fur-

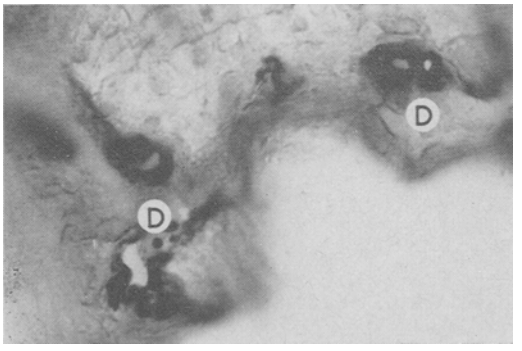

FIG. 3. Section of liver fluke stained with Oil Red O (x 1150). Fluke incubated for 12 hr in medium without glucose. Excretory ducts (D) contain neutral lipids only in the lining cells.

ther confirmed by color reactions with sulphuric acid), triglyceride, and free fatty acids. An unknown minor constituent with polarity less than that of triglyceride was noticed in some chromatograms (Fig. 1a) and had an Rf comparable with that of a short-chain cholesterol ester. However no color reaction was obtained at this spot with sulphuric acid, and the Rf value could have been consistent with the presence of a small amount of a glyceryl-ether diester (17). Polar lipids remained in the region of the origin in both of these solvent systems.

Separation of the total lipid fraction in solvent system 3 revealed a complex mixture of polar lipids (Fig. 1c). Tentative identification of some of the spots was made by comparison with standards, but more detailed work will be required before the identity of these components can be confirmed.

Histochemical Analysis of Lipids Within the Fluke

All the small excretory ducts in sections from flukes incubated for 12 hr in the glucose-enriched medium contained neutral lipids in both the lining cells and the lumina (Fig. 2). The lumina of most of the small ducts were completely filled with neutral lipids, in contrast to the large ducts where lipids appeared to be distributed only over the surfaces of the lining cells.

Flukes which had been deprived of glucose contained neutral lipids within the cells lining the excretory ducts. However most lumina did not contain demonstrable quantities of neutral lipid (Fig. 3).

Phospholipids could be seen within the lining cells of all ducts in sections from flukes which had been incubated with or without glucose. The lumina of some of the smallest ducts contained some phospholipid, but a weak color reaction was obtained in comparison with that produced within the lining cells. Phospholipids could not be detected within the lumina of the larger ducts.

Neither cholesterol (free or esterified), nor free fatty acids were demonstrable histochemically in any part of the sections from flukes incubated with or without glucose.

DISCUSSION

This work indicates that the liver fluke releases lipid through the excretory pores, that such lipid is a mixture far more complex than has been suggested by previous workers, and that important lipid constituents can be missed by complete reliance on comparatively insensitive histochemical techniques. In particular, earlier work (1) which reported the lack of excretion of phosphatides by the liver fluke was in error. The conclusion of von Brand (4) that simple release of exogenous food lipid is not involved may be correct but was based on a failure to observe cholesterol excretion. However the work of Clegg and Morgan (5) provided evidence to suggest that excretion of cholesterol and its esters does occur, and this has now been clearly demonstrated by the present authors. The question of excretion of exogenous lipid by the fluke is thus still open to investigation. Absence of histochemically demonstrable lipid material in other parts of the fluke suggests a lack of transport of lipids to the excretory ducts. Clarification of these points must await the results of a more detailed examination of lipid distribution within the fluke, and this work is in progress. These preliminary studies however do indicate that the cells lining the excretory ducts are the site of the synthesis of complex lipids which are excreted by the fluke.

Quantitatively the excretion of lipids by the fluke is impressive; it can be expressed as about 2% of the organism's wet weight per day or, alternatively, as about 10% of its lipid content during 4 hr of incubation. Consideration of the physiological significance of such high lipid excretion must be deferred until more detailed work has been completed. The greatly reduced excretion from ligated flukes however does suggest that release of lipids through the integument is a minor pathway of excretion if it is involved at all.

It is of interest that this small amount of lipid was still released into the medium by flukes with ligated oral and excretory openings. Although the amount of lipid thus obtained

was only about one-tenth of that from non-ligated flukes, the TLC patterns were identical. It has not yet been established whether such lipid excretion by ligated flukes is attributable to mobility of the lipid through the integument or to incomplete closure of mouths and excretory pores.

ACKNOWLEDGMENTS

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The Occurrence of Diastereomers of Phytanic and Pristanic Acids and Their Determination by Gas-Liquid Chromatography

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ABSTRACT

The methyl esters of phytanic (3,7,11,15-tetramethylhexadecanoic) and pristanic (2,6,10,14-tetramethylpentadecanoic) acids derived from phytol each can be resolved into two diastereomers by gas-liquid chromatography on an efficient open-tubular, gas-liquid chromatographic column with a polyester coating. Authentic D,D,D isomers prepared from the lipids of bacterium *H. cutirubrum* gave only one peak. In mammals the D,D,D isomers usually predominate, but in marine life the L,D,D isomers apparently are the principal forms. The origin and metabolic roles of the diastereomers are discussed.

INTRODUCTION

THE OCCURRENCE IN LIPIDS of mammals of phytanic (3,7,11,15-tetramethylhexadecanoic) acid and of the structurally related pristanic (2,6,10,14-tetramethylpentadecanoic) acid (1-9) has been investigated with particular emphasis on the potential role of rumen bacteria in converting phytol as the major source of phytanic acid (10-12). The accumulation of phytanic acid in human beings with Refsum's syndrome has led to further speculations as to the relative roles of exogenous and endogenous sources of this acid or of metabolic defects hindering its catabolism (13-25). In this rare condition phytanic acid can represent nearly half of the fatty acids of lipids in particular tissues (17). In ruminants phytanic acid may exceptionally be as high as 10% of the total fatty acids of serum lipids (4,25), but, although variable, the levels in other lipids are normally 0.01-0.2% of total fatty acids (3,7,25, 26). In nonruminants phytanic acid levels may be 0.01% or less of the total fatty acids. In reports for animal lipids, where pristanic acid proportions are given, these usually range from an equivalent amount down to one-tenth of phytanic acid level in the same sample (6,8,-25,26).

Three multiple-branched-chain fatty acids were suggested as commonly occurring components of lipids from higher marine organisms (27). The 4,8,12-trimethyltridecanoic acid, pristanic acid, and phytanic acid have been isolated from a marine oil and positively identified (28). Phytanic acid and other multiple-branched acids were also shown to be components of whale oil (29,30). The total of these three acids in marine lipids is moderately consistent at 0.5-1.5% of all fatty acids; phytanic acid predominates (26,27).

The occurrence of these three fatty acids may be conveniently studied without specific isolation through the use of open-tubular, gas-liquid chromatography (31). Further, samples of methyl phytanate and methyl pristanate from which interfering materials have been removed can be partially resolved into two components with butanediol-succinate coated, open-tubular columns of high efficiency. A number of samples of different origin have therefore been examined to evaluate this information from the point of view of origin and metabolic role of these acids.

EXPERIMENTAL

Gas-Liquid Chromatography

Columns were purchased from the Perkin-Elmer Corporation, Norwalk, Conn., and used without conditioning in a Perkin-Elmer Model 226 gas-liquid chromatograph, employing a flame ionization detector. The columns were of stainless steel tubing in a pancake configuration, 0.01 in ID and 150 ft long. Coatings were BDS (butanediol succinate polyester) or Apiezon L. Operating temperatures for these phases were respectively 150C and 190C; helium carrier gas was respectively at 40 and 50 psig. Injection port temperatures were 260C, and a No. 2 injection splitter was employed. Samples of approximately 1-5 μg of a particular ester were injected in neohexane solution with a Hamilton 10 μl microsyringe and were sufficiently small that all peaks were on scale at 2, 5, or 10 x attenuation.

Samples

The origin of samples analyzed in the present study is given in Table I. Materials of ter-

TABLE I

Origin of Samples of Phytanic and Pristanic Acids Examined in the Present Study with the Ratios of L,D,D and D,D,D Diastereomers Determined by Gas-Liquid Chromatography

Figure No.	Material	Origin	Reference	Ratio LDD/DDD form
1-A	Phytanic acid	Synthetic (from phytol)	Hansen (18)	0.94
1-B	Phytanic acid	Synthetic (from phytol)	Lough (4)	0.95
—	Phytanic acid	Sheep fat	Hansen (7)	0.95
1-C	Phytanic acid	Ox fat	Hansen (3)	0.75
1-D	Phytanic acid	Butterfat	Hansen (1)	0.48
1-E	Phytanic acid	Butterfat	Hansen (9)	0.53
1-F	Phytanic acid	Human (Refsum's Syndrome)	Hansen (17)	0.39
1-G	Pristanic acid	Sheep fat	Hansen (8)	0.67
1-H	Pristanic acid	Butterfat	Hansen (6)	0.96
4	Phytanic acid	Herring oil	Ackman[a] —	∽10
—	Phytanic acid	Cod liver oil	Ackman[a] —	∽10
—	Phytanic acid	Finwhale blubber	Ackman[a] —	∽ 2
4	Pristanic acid	Herring oil	Ackman[a] —	2.2
—	Pristanic acid	Cod liver oil	Ackman[a] —	1.2
—	Pristanic acid	Finwhale blubber	Ackman[a] —	0.65
2	Phytanic acid	Bacterial (all D,D,D)	Kates (32,33)	.....
2	Pristanic acid	Bacterial (all D,D,D)	Kates (32,33)	.....

[a] Current study.

restrial animal origin, with the exception of phytanic acid from sheep fat, were shown by open-tubular, gas-liquid chromatography to contain only 1-5% of various impurities. These minor components individually did not exceed 1% of net area response. The phytanic acid from sheep fat was in a concentrate containing 28.7% phytanic acid and 58.7% *ante-iso* heptadecanoic acid, with various minor impurities. None of the latter interfered with the determination of isomer ratios.

Authentic 3D,7D,11D,15-tetramethylhexadecanoic acid, containing about 5% of 2D,6D,-10D,14-tetramethylpentadecanoic acid, was prepared by M. Kates from the di-*O*-phytanyl glyceryl ether moiety of lipids in *Halobacterium cutirubrum,* as described elsewhere (32); the pristanic acid impurity was derived from the phytanic acid during the preparation. The configuration of the phytanic and pristanic acids was established as "all D" by conversion to compounds of known configuration (33).

Two samples of phytanic acid were synthesized from phytol of plant origin (I-A, I-B; Table I). Concentrates of branched-chain fatty acid methyl esters from herring, cod liver, and finwhale oils were prepared from hydrogenated samples by urea-complex treatment. Phytyl acetate was prepared from commercial phytol and hydrogenated to give dihydrophytyl acetate.

RESULTS

Of the BDS polyester columns received from the manufacturer, only one out of four which were examined possessed the requisite number of theoretical plates (ca. 40,000 with methyl palmitate) for resolution of the diastereomers of multiple-branched fatty acids. This particular column was employed throughout the study. The efficiency of the remainder (20,000-25,000 plates) was such that peaks for these materials were broader than those for neighboring homogeneous components (e.g.,

TABLE II

Gas-Liquid Retention Data for L,D,D and D,D,D Diastereomers on a Butanediol-Succinate Coated, Open-Tubular Column at 150C and with Helium at 40 psig as Carrier Gas[a]

Material	Ret. Time (rel. to 16:0)	Equivalent chain length
Methyl myristate	0.443	14.00
Methyl 4D,8D,12-trimethyltridecanoate	0.470	14.12
Methyl 2L,6D,10D,14-tetramethylpentadecanoate	0.910	15.76
Methyl 2D,6D,10D,14-tetramethylpentadecanoate	0.921	15.80
Methyl palmitate	1.000	16.00
Methyl 3L,7D,11D,15-tetramethylhexadecanoate	1.50	16.98
Methyl 3D,7D,11D,15-tetramethylhexadecanoate	1.52	17.02
3L,7D,11D,15-tetramethylhexadecanol acetate	2.22	17.93
3D,7D,11D,15-tetramethylhexadecanol acetate	2.25	17.97

[a] Methyl palmitate emerged 28 minutes after the solvent front. The ECL data were obtained from extrapolation of the methyl myristate-methyl palmitate line.

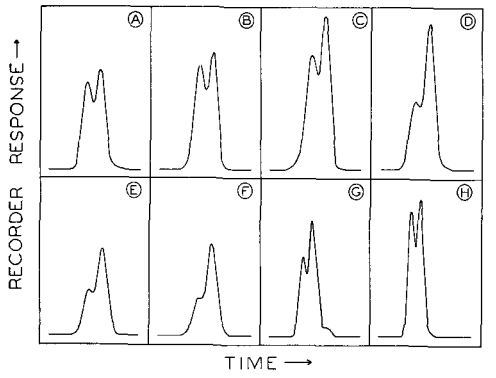

FIG. 1. Partial gas-liquid chromatograms of methyl esters of phytanic (A-F) and pristanic (G and H) acids of synthetic or mammalian origin as identified in Table I.

methyl palmitate), but there was no obvious resolution. Only one Apiezon L column was investigated, and, although under the operating conditions which were specified the number of theoretical plates was the same as with the better BDS columns (ca. 41,000 with methyl palmitate), there was only slight evidence for the resolution of diastereomers. This is a common relative property of these GLC substrates; separation factors for *iso* and *ante-iso* heptadecanoates are 1.04 for the Apiezon phase and 1.07 for the polyester phase. The poorer performance of Apiezon and SE-30 nonpolar phases in this respect has been illustrated elsewhere (31,34).

The virtue of the Apiezon column was that analyses on it made sure that there were no superimposed materials which would affect the

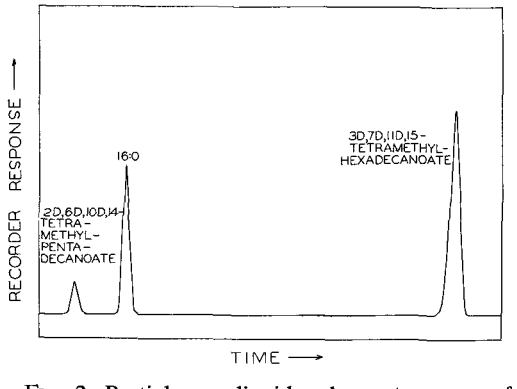

FIG. 2. Partial gas-liquid chromatogram of methyl ester of D,D,D phytanic acid (with pristanic D,D,D acid impurity) prepared from lipids of *Halobacterium cutirubrum*. Methyl palmitate added as a marker.

ratios of diastereomers obtained on the BDS column. Methyl phytanate is usually found on polar columns in the immediate vicinity of methyl heptadecanoate (12,19,31), as shown by the equivalent chain-length (ECL) data of Table II. Retention data were obtained by measuring distances from the leading edge of the solvent peak to the intercepts of the frontal tangents and baselines. Data for the L,D,D isomers from chromatograms of synthetic and naturally occurring phytanic acids (Table I, Fig. 1) agreed in all cases and were measured directly. Data for the D,D,D, isomers were measured directly from chromatograms of the pure D,D,D isomers (Fig. 2). Approximate retention data for the components of longer retention time agreed with those for the reference D,D,D isomers, and chromatograms of mixed esters showed exact coincidence of the D,D,D isomers and these components (Figs. 3 and 4). Retention data obtained by measuring to the peak apices were also in good agreement when the column loads were small (cf. 35).

In samples of marine origin only one component could be detected in the peak (Fig. 4) considered to be methyl 4,8,12-trimethyltridecanoate on the basis of calculated ECL values (31,36), literature data (28), GLC behavior on polar and nonpolar phases (31), and nonadductive behavior on repeated treatments with urea. The width of this peak was similar to that of the neighboring methyl myristate peak. Similarly the width of the 2D,6D,10D,14-tetramethylpentadecanoate peak was similar to that of the neighboring methyl palmitate (Fig. 2).

The pristanic and phytanic acids of marine origin have not been defined by isolation in a form substantially free from other materials. Identifications in concentrates were based on complete coincidence with mammalian samples on both BDS and Apiezon phases, quantitative interrelationships on both phases with a mutual internal standard, and the lack of change in the relative proportions on repeated urea-complex treatment. Dihydrophytyl acetate was also partially resolved into two components (Fig. 5).

Ratios of diastereomers (Table I) were based on peak height measurements, corrected in the case of the D,D,D isomers for tailing of L,D,D isomers in proportion to peak height. The correction was based on observed tailing of the D,D,D isomer. In phytanic acid of marine origin the proportion of D,D,D isomer was so low as to render correction meaningless, and ratios given are approximations only. The L,D,D isomer peak apex was in advance of the leading edge of the D,D,D isomer.

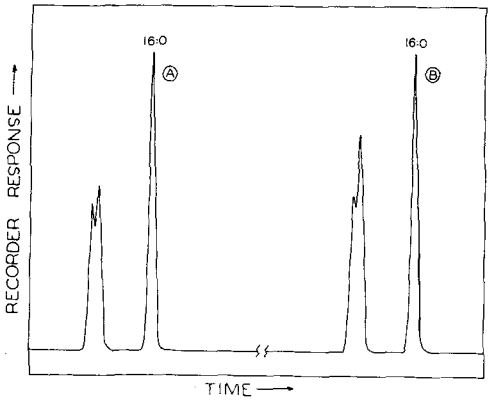

FIG. 3. Partial gas-liquid chromatograms of methyl ester of pristanic acid from butterfat (A), with the addition of authentic D,D,D pristanic acid (B). Methyl palmitate added as a marker.

DISCUSSION

Four racemates of methyl esters of 2,4,6-trimethyloctanoic acid have been synthesized and resolved on an open-tubular column, coated with polypropylene glycol (37). Three of the four racemates were completely resolved, the fourth separated only partially from a diastereomeric form. In this type of multi-methyl-branched fatty acid, occurring naturally in the preen glands of swans, the optically active acids observed are "all D" in structure. In the course of synthesis of these acids stereospecificity was observed in a dealkylation step (37).

The results of the present GLC analyses (Table I, Fig. 1) are sufficiently diverse in terms of isomer ratios of L,D,D and D,D,D diastereomers for phytanic and pristanic acids to explain the differing optical activities observed in samples isolated from mammalian sources. The ratios are qualitatively in agreement with optical rotations for those samples where determinations were made at common wavelengths (11). Most of the optical activity originates in the 2- and 3-methyl substituents; there is much less contribution from centrally located methyl substituents (37,38; cf. 39,40).

The GLC results for isomer ratios (Table I) are not completely satisfactory since the separation of diastereomers was incomplete and area proportions could not be obtained. Peak height adjustment of the D,D,D isomer for tailing of the L,D,D isomer did not raise the proportions for phytanic acids derived from phytol to the expected 1:1 ratio. It is not known if this is because of insufficient correction, slight stereospecificity in the reduction

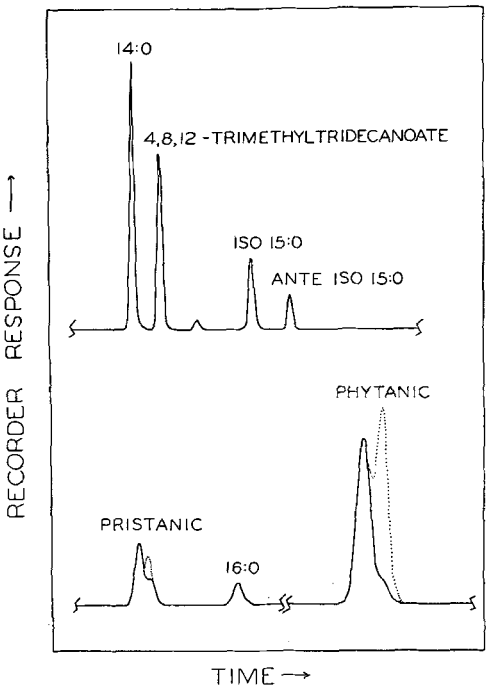

FIG. 4. Partial gas-liquid chromatogram of hydrogenated methyl esters of herring oil fatty acids after urea complex treatment. Dotted peaks show co-chromatogram with methyl esters of authentic D,D,D pristanic and phytanic acids.

(cf. 37), a GLC effect which might broaden the L,D,D isomer, or the presence of an impurity originating in the phytol. The same ratio of peak heights was evident in the dihydrophytyl acetate chromatogram (Fig. 5).

It is known that, in ruminants, phytanic acid is produced by rumen bacteria (10-12); it then passes into the circulatory system and lipid pool. Production of pristanic acid in ruminants is presumably concurrent with the oxidation step in which the phytol alcohol group is converted to the carboxyl group. It has been shown that phytanic acid can be produced in non-

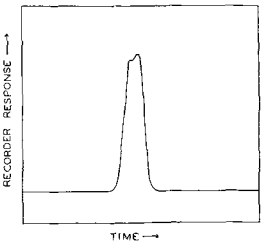

FIG. 5. Partial gas-liquid chromatogram of dihydrophytyl acetate.

ruminants from ingested phytol (15,16,19-24), and that, in human beings with Refsum's syndrome, catabolism of phytanic acid is slower than in normal human beings (16) and can involve an unusual metabolic pathway (22).

From the data in Table I it might be inferred that, in ruminants, there is either partially stereospecific formation of D,D,D isomers or preferential catabolism of L,D,D isomers. The ratio of 0.96 for one pristanic acid (from butterfat) suggests that catabolism is the key reaction since it is possible that the material in this sample originated during a period of heavy feeding and passed into the milk before much pristanic acid could be catabolized. Similarly the two mammalian phytanic acid samples of high isomer ratios (from sheep fat and ox fat) are from fats where deposition of metabolically inactive fatty acids would be likely in periods of heavy feeding. This is speculation only.

The markedly low ratio for phytanic acid isomers in the sample from a human being with Refsum's syndrome can also be interpreted as evidence for a relatively sluggish catabolism of the D,D,D isomer compared to the L,D,D isomer. However this preference may only be a slight amplification of a normal process in mammals favoring catabolism of the L,D,D isomer. Alternatively in mammals endogenous synthesis, proposed as one source of this acid, may be stereospecific and lead to the D,D,D isomer (41; cf. 24).

Ratios of diastereomers of the samples examined thus do not provide clear-cut evidence on the question of the endogenous or exogenous origin of phytanic acid involved in Refsum's syndrome. However ratios of the diastereomers could provide a useful tool for further investigations, based on catabolism of phytanic acid samples of specific structure.

The isomers from marine animals occur in proportions completely different from those in mammals although the authors believe that phytol in marine chlorophyll is identical with that of terrestrial origin. In higher marine lifeforms, such as herring and cod, the less common fatty acids represent a pooling of fatty acids that originate in lower forms of life since marine lipids, excepting those synthesized by phytoplankton, are recycled continuously through the food chains. The presumed specific occurrence of 4D,8D,12-trimethyltridecanoic acid is satisfactorily explained by assuming that this acid is derived from phytol through oxidation of intermediate 1,3-phytadienes (42) or of the principal "zamene" (2,6,10,14-tetramethyl-2-pentadecene) (43).

H. cutirubrum is a marine bacterium favoring a high salt concentration (e.g., tidal pools, solar salt ponds). Although it produces specifically a D,D,D phytanyl skeleton (33), there is no reason to assume that this would be a characteristic of marine bacteria in general (44). Bacteria play a significant role in the marine food chain (45), but it is unlikely that the amount of phytanic acid involved would be large in respect to that originating in phytol. There are relatively high levels of hydrocarbons, particularly pristane, in some species of zooplankton important in the aquatic food chain (46-48). To explain the preponderance of L,D,D-phytanic acid, it is suggested that the route from phytol to pristane proceeds through phytanic acid (48,49) with preferential conversion of the D,D,D-phytanic acid to pristane, leaving the L,D,D isomer to accumulate in depot fats of higher species ingesting the zooplankton. In proportion to lipid the pristane content of *Calanus* (48) is very similar to that of phytanic acid in higher species (27,31).

In Nova Scotia water, finwhales feed primarily on zooplankton and occasionally on small fish; the food intake thus does not differ greatly from that of herring (50). Finwhale blubber therefore offered an opportunity to investigate the phytanic acid ratio in a mammal which was presumably ingesting primarily the L,D,D isomer. The ratio of the two phytanic acid isomers could not be determined as exactly as in the fish since there was evidence, in two different samples, of a small amount of a third material partly under the D,D,D isomer. However the ratio was notably lower than in the fish oil samples, as was the ratio for pristanic acid (Table I). The finwhale results support the suggestion that intake is largely L,D,D phytanic acid, but the greater proportion of D,D,D isomer could arise from a mammalian characteristic favoring either endogenous synthesis of the D,D,D isomer or catabolism of the L,D,D isomer as discussed above.

Marine oils originating in fish thus appear to offer a source of phytanic acid specifically enriched in the L,D,D isomer. Marine origins have been suggested for petroleum, but several isoprenoid fatty acids isolated from a California petroleum showed little or no optical activity (51). If of marine origin, then these may be derived from phytol and related hydrocarbons (48) and not from lipids of marine crustacea and vertebrates or from marine bacteria (52).

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Gas-Liquid Chromatography of Triglyceride Mixtures Containing Both Odd and Even Carbon Number Fatty Acids[1,2]

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ABSTRACT

Quantitative GLC of triglycerides has been extended to natural fats containing both odd and even carbon number fatty acids. A 1.83-m glass column containing 3.0% JXR silicone on 100/120 mesh Gas-Chrom Q resolved triglycerides differing by only one carbon number. Peak resolution was significantly improved by hydrogenating each triglyceride sample prior to GLC analysis.

The triglycerides of four fish oils (mullet, tuna, menhaden, and pilchard) and one seed fat (*Acanthosyris spinescens*) containing odd carbon number fatty acids were analyzed by this technique. The method was also useful for determining the triglyceride composition of the cyclopentene fatty acid oil from *Hydnocarpus wightiana* seeds.

INTRODUCTION

ALTHOUGH MOST FATTY ACIDS occurring in nature contain an even number of carbon atoms, GLC has clearly demonstrated that odd carbon fatty acids are also present in most natural fats (1-3). Seed fats usually contain only traces of straight-chain, odd carbon fatty acids, but many fish oils contain appreciable amounts (1-5%). Mullet oil (3,4) and *Acanthosyris spinescens* seed fat (5,6) are unusual for they contain major amounts (>10%) of such odd carbon number fatty acids. Branched-chain, odd carbon number fatty acids are also found in many animal and bacterial fats (7,8). The presence of both odd and even carbon fatty acids in a natural fat inevitably leads to triglycerides of odd carbon number. However present methodology cannot resolve triglycerides differing by only one carbon atom.

Although GLC has been widely used for the analytical separation of triglycerides by molecular weight, quantitative techniques have only been capable of resolving triglyceride molecules differing by two carbon atoms (9,10). During the GLC analysis of tuna muscle triglycerides (11) the appearance of small partially-resolved peaks in between the larger carbon number 44, 46, 48, and 50 peaks was noted (Fig. 1). Since odd carbon number fatty acids were known to be present in tuna triglycerides (11), the small in-between peaks were presumably odd carbon number triglycerides. Kuksis, McCarthy, and Beveridge (12) have also noted that the even carbon number triglycerides of butter oil are incompletely resolved during GLC, indicating the probable presence of odd carbon number triglycerides. The authors have previously shown (9) that triglycerides of carbon numbers 45, 46, 47, and 48 could be resolved on a 1.52-m silicone GLC column, but calibration factors in these early experiments were too high for accurate quantitation.

This paper describes a method for the quantitative analysis of odd carbon number triglycerides by using high-resolution GLC columns. Four fish oils and one seed fat containing appreciable odd carbon fatty acids have been analyzed for triglyceride composition by using this new technique. Possible applications to triglycerides containing cyclic fatty acids are also discussed.

PROCEDURES

Materials

Trilaurin, trimyristin, tripalmitin, and tristearin calibration standards of greater than 99% purity were purchased from Applied Science Laboratories, State College, Pa. Triolein, triarachidin, and tribehenin standards of 99% purity were purchased from the Hormel Institute, Austin, Minn. A synthetic mixture of C_{45}, C_{46}, C_{47}, and C_{48} triglycerides was prepared by reacting equal molar quantities of pentadecanoyl and palmitoyl chlorides with glycerol as described by Hartman (13).

Two samples of mullet (*Mugil cephalus*) were analyzed. Sample A was supplied by

[1]Presented at the AOCS meeting, New Orleans, May, 1967.

[2]Abbreviations: GLC = gas-liquid chromatography, TLC = thin-layer chromatography, DEGS = diethylene glycol succinate polyester.

Donald Moore of the U.S. Bureau of Commercial Fisheries, Galveston, Tex. These mullet were caught during December 1965 in coastal waters near Galveston and were 13-16 cm in length. The head, intestines, and fins were removed from six fish (140 g total live weight) leaving 75 g of muscle tissue, which was then extracted by using the $CHCl_3/CH_3OH$ method of Bligh and Dyer (14). The muscle from these six fish yielded 1.5 g of crude lipid (2.0%). Mullet sample B was provided by Hermann Schlenk, Hormel Institute, Austin, Minn. This oil was obtained from mullet caught off the coast of Bangs Island near Pascagoula, Miss. in early May 1965. The fish averaged 50 g in weight and were 25-30 cm long. Forty pounds of whole mullet were steam-rendered to yield 3.2% crude lipid on a wet-weight basis.

The origins of "little tuna" muscle oil (*Euthynnus alletteratus*) and menhaden body oil (*Brevoortia tyrannus*) have been described previously (11).

Pilchard oil (*Sardinops caerulea,* Girard) of North Pacific origin was supplied by R. G. Ackman, Fisheries Research Board of Canada, Halifax, Nova Scotia. This sample was a commercial, alkali-refined oil produced by the U.S. Department of Interior, Fish and Wildlife Service, Seattle, Wash. Fatty acid composition and other data on this oil have been reported by Ackman (15).

A sample of *Acanthosyris spinescens* oil was provided by R. G. Powell and C. R. Smith Jr., Northern Regional Research Laboratory, Peoria, Ill. The unusual C_{17} acetylenic acids and other fatty acids of this oil have been described by Powell et al. (5,6).

Hydnocarpus wightiana seed fat was provided by P. K. Raju of this institution. The fat had been obtained by petroleum ether extraction of seeds obtained from western India.

Hydrogenated *Sterculia foetida* seed fat was obtained from Hermann Schlenk, Hormel Institute, Austin, Minn. This oil had been hydrogenated by using the procedure of Gellerman and Schlenk (16) so that all cyclopropene and cyclopropane rings had been broken to yield either straight-chain or methyl-branched fatty acids.

Methods

Preparation of Triglycerides. Each fat except *Sterculia foetida* was fully hydrogenated by the method of Farquhar et al. (17), using freshly distilled dioxane as a solvent in place of ethanol. The triglycerides were isolated from each of the fats with preparative TLC on 1.0-mm thick plates of Adsorbosil-1 developed in 79:20:1 petroleum ether-diethyl ether-acetic acid. The triglyceride fraction was the major neutral lipid component in all the crude fat samples. Since *Acanthosyris spinescens* seed fat contains 20% hydroxy fatty acids, only its nonhydroxy triglycerides were isolated for GLC analysis.

GLC of Fatty Acids. Methyl esters were prepared from the hydrogenated fats after TLC purification. Normal H_2SO_4-catalyzed methanolysis (18) was carried out in the stopcock ampules described by Archibald and Skipski (19). After extraction the methyl esters were analyzed by using a 1.83 m × 2.4 mm ID column containing 20% DEGS polyester coated on 60/80 mesh Chromosorb W as previously described (18). Peaks were identified by co-chromatography with known materials, by plotting carbon number vs. log of retention time and by comparison with compositions reported by other workers. Branched-chain acids were identified as those peaks which did not correspond to the elution times of a homologous series of straight-chain methyl esters. Peak areas were determined by mechanical integration and triangulation. All fatty acid compositions are reported in mole percentage.

GLC of Triglycerides. An F&M Model 400 gas chromatograph, equipped with a hydrogen flame detector and automatic temperature programming, was used for GLC of triglycerides. Operating conditions were the subject of numerous experiments, which are described in detail in the Experimental section. Optimum operating conditions are listed in Table II.

Triglyceride peaks were identified by co-chromatography with known standards. Peak areas were determined by triangulation. Quantitative response factors for the various carbon number triglycerides were determined from known composition mixtures of trilaurin, trimyristin, tripalmitin, tristearin, triarachidin, and tribehenin. The f_m values [molar calibration factors determined by the internal normalization technique (9)] of the simple triglycerides in the calibration mixtures were then plotted vs. carbon number, and f_m values for all carbon numbers were interpolated from the resulting graph. All triglyceride compositions are reported in mole percentage.

EXPERIMENTAL SECTION

Operating Conditions

Since initial results had already indicated that odd carbon number triglycerides can be

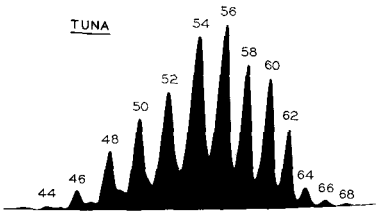

FIG. 1. Gas chromatogram of hydrogenated tuna muscle triglycerides. Operating conditions: F&M 400 gas chromatograph; 0.53 m x 2.4 mm ID stainless steel column packed with 3.0% JXR on 100/120 mesh Gas Chrom Q; column programmed 200-360C at 4.0C/min with 100 ml/min. He carrier gas; flash heater at 350C; detector base at 310-350C.

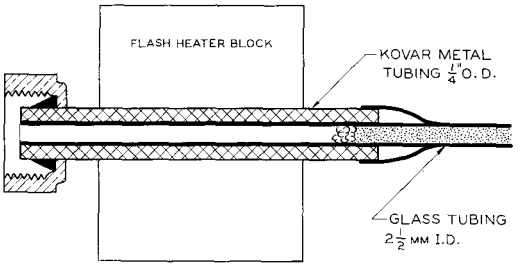

FIG. 2. Construction of Kovar glass-to-metal seal used at ends of the GLC column. This seal allowed glass columns to be used to 400C with a leak-free connection at each end.

partially separated by GLC (Fig. 1), the major problem was to change the operating conditions to improve peak resolution as much as possible. The first logical step was to increase column length, but this increased elution temperatures. Well-conditioned JXR liquid phase could not be used above 375C without excessive baseline rise. Comparison of 1.0%, 2.0%, and 3.0% JXR columns indicated that 3.0% JXR was the lowest level of liquid phase which can be used without peak tailing that causes a considerable decrease in resolution. Experiments demonstrated that a 1.83-m, 3.0% JXR column eluted tribehenin at 375C, and this column length was adopted for all further work.

The authors have demonstrated in a previous paper (9) that helium carrier gas gives significantly better resolution of triglyceride GLC peaks than nitrogen. Therefore helium carrier gas was used in all the current experiments.

It has been shown that glass columns give significantly better resolution than stainless steel columns in GLC of triglycerides (9). But in the first attempts to use glass columns up to 400C, the authors were unable to maintain a leak-free, glass-to-metal seal at the ends of the column. Either Teflon ferrules or silicone rubber O-rings (9) are normally used for sealing a glass GLC column in a metal tubing fitting, but neither of these materials will maintain a gas-tight seal above 300C for any length of time. To overcome this difficulty, the special Kovar glass-to-metal seals described by Radin (20) were used. These seals are leak-free at temperatures as high as 400C but are quite fragile and will not withstand the torque required for connecting tubing fittings. This limitation is no problem however if the U-tube column is made in two halves, which are connected together after they are installed in the gas chromatograph. The column is then packed while in place in the chromatograph.

Figure 2 shows the design of the Kovar glass-to-metal seal used in the present work. A Kovar metal rod was drilled to fit as a tight sleeve over the glass tubing of the column. A Swagelok tubing fitting was installed at one end of the rod, and a Kovar glass-to-metal seal was constructed at the other end. Figure 2 shows such a Kovar seal placed in the flash heater at the entrance to the column. A similar installation was made at the column exit. The Kovar glass-to-metal seal was designed so that, with on-column injection, the sample came in contact with glass only. When exposed Kovar metal was used in the flash heater zone, considerable degradation of triglycerides occurred. Kovar seals, such as shown in Figure 2, can be made in any well-equipped glass-blowing shop.

The preparation of longer columns stable to 375C requires more extensive conditioning than with shorter columns. Therefore the 1.83-m JXR columns were conditioned for 4 hr at 350C, 1 hr at 375C, and 1 hr at 400C before use. The column was then used in normal pro-

TABLE I

Typical Calibration Factors and Elution Temperatures for Simple Triglycerides

Triglyceride	Carbon number	Weight calibration factor	Molar calibration factor	Elution temperature
		f_w	f_m	°C
Trilaurin	36	1.00	1.00	275-283
Trimyristin	42	0.89	0.79	300-308
Tripalmitin	48	0.90	0.72	320-327
Tristearin	54	1.06	0.76	340-347
Triarachidin	60	1.56	0.98	353-360
Tribehenin	66	1.90	1.08	368-375

Operating conditions same as listed in Table II.

TABLE II
Optimum Operating Conditions for High-Resolution Gas-Liquid Chromatography of Triglycerides

Gas chromatograph	Instrument designed for steroid analysis, equipped with flame detector and temperature programming
Column	1.83 m x 2.5 mm ID glass with Kovar glass-to-metal seal at each end
Packing	3% JXR on 100/120 mesh Gas Chrom Q
Column conditioning	4 hrs at 350C, 1 hr at 375C, and 1 hr at 400C
Carrier gas	100 ml/min helium
Column temperature	210→375C at 4.0C/min
Injection heater temperature	350C
Detector base temperature	320-360C

grammed runs for 5-10 days before stable calibration factors were obtained. Typical weight and molar calibration factors (9) for a well-conditioned column are listed in Table I. It is apparent that some losses occur with carbon numbers above 45; but since a linear relationship exists between peak area and amount of sample injected (9,11), these losses can easily be compensated for by proper calibration procedures. Typical elution temperatures for the various carbon numbers are also listed in Table I. Elution temperatures decrease slightly with column age.

During early experiments the authors often observed a series of regularly spaced, extraneous, minor peaks eluting between 200 and 300C. These peaks appeared even when no sample was injected and were finally traced to septum bleed, i.e., material vaporized out of the septum as oven temperature increased. This problem has been discussed by Kolloff (21) and also in a recent paper from this laboratory (9). These extraneous peaks were eliminated by laminating two septums together. The outside septum was changed frequently to

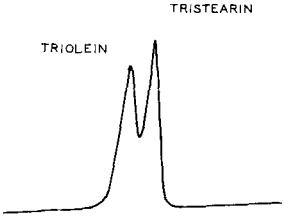

FIG. 3. Gas chromatogram showing partial separation of triolein and tristearin on a 1.83-m,3.0% JXR column. Operating conditions listed in Table II.

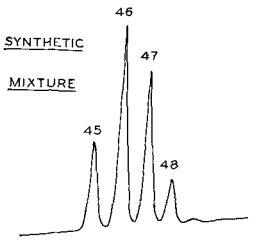

FIG. 4. Gas chromatogram of a synthetic mixture of C_{45}, C_{46}, C_{47}, and C_{48} triglycerides on a 1.83-m, 3.0% JXR column. Operating conditions listed in Table II.

maintain a gas-tight seal; and a well-conditioned septum was used on the inside facing the column. In addition, it was found useful to wrap the septum nut with one turn of stainless steel tubing through which tapwater was circulated. This arrangement successfully lowered septum temperature and helped eliminate the extraneous peaks without interfering with sample vaporization.

Optimum operating conditions for high-resolution GLC of triglycerides are summarized in Table II. A synthetic mixture of C_{45}, C_{46}, C_{47}, and C_{48} triglycerides (Figure 4) was separated with baseline resolution between the four peaks, indicating that the operating conditions which were selected successfully resolve triglycerides differing by only one carbon number.

TABLE III
Fatty Acid Composition of Fully Hydrogenated Fish Oils (*Mole %*)

Fatty acid[a]	Mullet A muscle	Mullet B body	Tuna muscle	Menhaden body	Pilchard body
10	tr	—	—	—	tr
11	tr	—	—	—	tr
12	0.2	0.1	0.1	0.2	0.1
branched	tr	tr	—	—	0.1
13	0.3	0.3	0.2	—	0.1
branched	tr	0.1	—	—	tr
14	6.2	6.0	4.7	10.5	9.0
branched	0.5	0.4	0.1	0.2	0.2
15	8.7	6.7	1.1	0.7	0.6
branched	0.1	0.2	0.1	0.1	0.1
16	41.3	38.9	31.8	37.3	31.3
branched	0.2	0.2	2.4	0.1	0.1
17	8.5	7.3	2.0	1.2	0.9
branched	—	—	0.3	0.1	—
18	12.9	15.9	21.2	21.5	18.5
19	2.1	1.6	0.4	0.4	0.2
20	12.1	11.9	9.1	14.8	20.2
21	0.7	0.8	0.4	0.6	0.6
22	5.9	9.5	25.0	11.3	16.7
23	0.1	tr	0.1	0.1	tr
24	0.2	0.1	1.0	0.9	1.3

[a]Listed in order of elution on DEGS polyester GLC column.

TABLE IV
Fatty Acid Composition of Fully Hydrogenated Vegetable Fats (Mole %)

Fatty acid[a]	*Acanthosyris spinescens*	*Hydnocarpus wightiana*	*Sterculia foetida*
unknown	—	0.2	—
14:0	—	—	0.2
15:0	—	—	0.1
14:C[b]	—	0.3	—
16:0	3.7	3.8	17.5
15:C	—	0.5	—
17:0	30.2	—	1.4
16:C	—	46.4	—
branched[c]	—	—	7.2
18:0	65.7	3.5	11.1
branched[d]	—	—	46.9
19:0	—	—	15.6
18:C	—	45.3	—
20:0	0.4	—	—

[a]Listed in order of elution on DEGS polyester GLC column
[b]C = cyclopentane fatty acid
[c]8(9) – methylheptadecanoic acid
[d]9(10) – methyloctadecanoic acid

Sample Hydrogenation

Early experiments showed that complete hydrogenation of triglyceride samples before GLC analysis greatly improved resolution. This is illustrated by the partial resolution of triolein and tristearin on a 1.83-m, 3% JXR column as shown in Fig. 3. Hydrogenation of the triolein-tristearin mixture would produce a single peak. All samples were completely hydrogenated before analysis to improve peak resolution and to avoid thermal decomposition

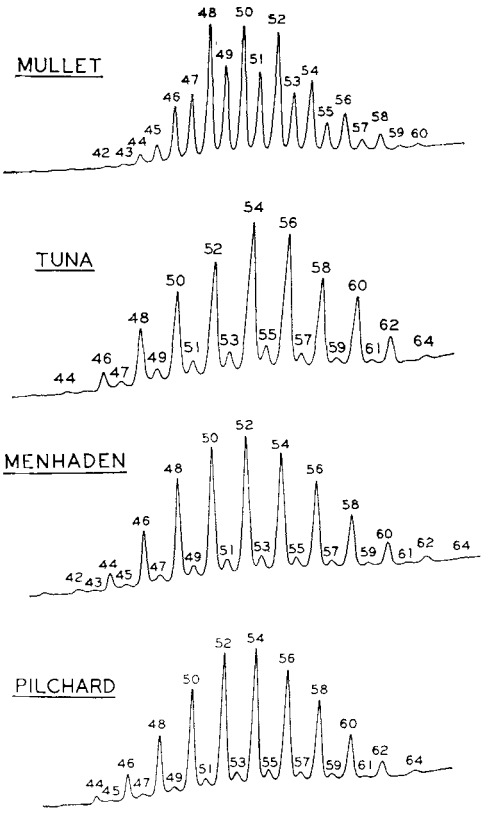

FIG. 5. Gas chromatograms of fully hydrogenated mullet, tuna, menhaden, and pilchard oil triglycerides on a 1.83-m, 3.0% JXR column. Operating conditions listed in Table II.

TABLE V
Triglyceride Composition of Fully Hydrogenated Fats (Mole %)

Carbon number	Mullet A muscle	Mullet B body	Tuna muscle	Menhaden body	Pilchard body	*Acanthosyris spinescens*
42	0.1	0.2	tr	0.4	tr	—
43	0.2	0.1	—	tr	—	—
44	0.8	0.6	0.3	1.4	0.7	—
45	1.4	1.0	0.2	0.4	0.2	—
46	4.5	3.7	1.5	5.0	2.4	—
47	5.7	4.0	0.6	0.8	0.4	—
48	12.2	9.8	4.9	9.9	5.7	—
49	8.3	6.2	1.2	1.2	0.6	—
50	12.5	12.2	8.2	13.4	10.5	1.8
51	8.0	6.5	1.5	1.5	1.1	9.2
52	13.6	13.8	11.0	16.3	15.0	22.1
53	6.7	5.9	2.0	1.6	1.3	32.5
54	9.0	10.8	17.5	15.3	16.6	34.4
55	4.0	3.9	2.2	1.3	1.3	—
56	5.7	8.5	17.1	12.3	15.9	—
57	2.0	2.2	1.5	1.0	1.2	—
58	3.0	5.2	12.1	9.3	13.2	—
59	0.8	1.1	1.0	0.6	0.8	—
60	1.1	2.7	11.0	5.4	8.3	—
61	0.2	0.3	0.6	0.2	0.3	—
62	0.2	1.1	4.8	2.2	3.5	—
63	—	tr	0.1	tr	0.1	—
64	—	0.2	0.7	0.5	0.9	—

TABLE VI
Average Fatty Acid Chain Length

Oil	Calculated from methyl ester data	Calculated from triglyceride data
Mullet A *muscle*	17.08	17.07
Mullet B body	17.37	17.33
Tuna muscle	18.34	18.31
Menhaden body	17.61	17.67
Pilchard body	18.13	18.13
Acanthosyris spinescens	17.63	17.63

of the highly unsaturated fatty acids found in fish oils. No information was lost by hydrogenation since GLC separations are on the basis of carbon number only.

Applications

Eight different natural fats containing odd carbon or cyclic fatty acids (Tables III and IV) were selected for triglyceride analysis by the technique described above.

Five fully hydrogenated fish oils (mullet A, mullet B, tuna, menhaden, and pilchard) were analyzed for triglyceride composition by GLC. The resulting chromatograms are shown in Figure 5, and the triglyceride compositions by carbon number are reported in Table V. Peak resolution was quite satisfactory for accurate quantitation in each case. Even the pilchard triglycerides containing only 2.4% odd carbon fatty acids (and thus a maximum of 7.2% odd carbon number triglycerides distributed between 10 peaks) gave small but distinctly separated odd carbon number triglyceride peaks.

Nonhydroxy triglycerides from hydrogenated *Acanthosyris spinescens* seed fat contain 30.2% 17:0 fatty acid. These triglycerides were analyzed by GLC and were cleanly resolved into five peaks (Figure 6). Quantitative results are reported in Table V.

The average fatty acid chain-length was calculated from the triglyceride composition for each of the fish oils and the *Acanthosyris spinescens* by using the method of Kuksis et al. (12). These values show close agreement with the average fatty acid chain-lengths which were calculated from methyl ester GLC data (Table VI), indicating that the carbon number distributions reported in Table V are close to the correct values.

The availability of a column for high-resolution GLC of triglycerides prompted also an attempt at analysis of natural fats containing cyclic fatty acids. Zeman and Pokorny (22) have shown that cyclopentene fatty acids have a much longer elution time than their straight-chain analogues on a nonpolar Apiezon col-

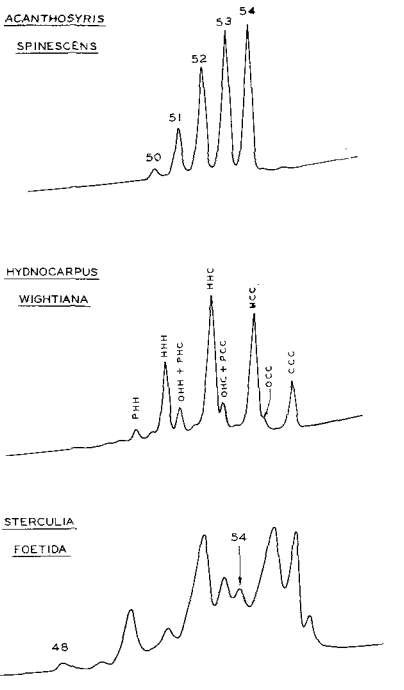

FIG. 6. Gas chromatograms of fully hydrogenated, nonhydroxy triglycerides from *Acanthosyris spinescens, Hydnocarpus wightiana,* and *Sterculia foetida* seed fats on a 1.83-m, 3.0% JXR column. Operating conditions listed in Table II. P = palmitic acid; O = oleic acid; H = hydnocarpic acid; C = chaulmoogric and gorlic acids; OHH = oleodihydnocarpin; etc.

umn. The question was whether cyclopentane triglycerides could be separated from their straight-chain analogues on a 1.8-m JXR silicone column. Fully hydrogenated *Hydnocarpus wightiana* triglycerides were chromatographed under optimum operating conditions, and the resulting chromatogram is shown in Figure 6. Peaks were tentatively identified based on the fatty acid composition of the original triglyceride sample. It is apparent that two distinct families of peaks were resolved. One family of peaks (HHH, HHC, HCC, and CCC) contains three cyclopentane acids per triglyceride molecule; and the other family (PHH, OHH + PHC, OHC + PCC, and OCC) contains two cyclopentane acids and one straight-chain acid per triglyceride molecule. Other minor peaks are also present. Resolution of the two families of peaks is incomplete, but approximate analyses can be made.

Sterculic acid contains 19 carbon atoms. Although its cyclopropene group is not amen-

able to GLC, sterculic acid can be quantitatively converted to a mixture of n-nonadecanoic and 9(10)-methyloctadecanoic acids by properly controlled hydrogenation (16). *Sterculia foetida* seed fat was hydrogenated in this manner, and its triglycerides were analyzed by high-resolution GLC (Figure 6). The resulting peaks are of varying width and are insufficiently separated for proper identification or quantitation.

DISCUSSION

Analytical Technique

The optimum operating conditions described in Table II now make it possible to determine the carbon number distribution of natural fat triglycerides containing straight-chain, odd carbon number fatty acids.

Branched-chain acids of even or odd carbon number tend to decrease resolution in triglyceride GLC. Abrahamsson et al. (25) have shown that *x*-methyloctadecanoate is eluted considerably before an n-nonadecanoate on a silicone GLC column, regardless of the position of the methyl branch in the fatty acid chain. If this is true for branched-chain fatty acid methyl esters, it is likely to also hold for branched-chain triglycerides on a JXR silicone column. Therefore 16-methylheptadecano-distearin would undoubtedly elute before tristearin even though both triglycerides have the same number of carbon atoms. This peak-broadening effect of branched-chain fatty acids will obviously decrease resolution significantly. The fish oil triglycerides which were analyzed contain such a small amount of branched-chain acids that satisfactory GLC resolution of odd carbon number triglycerides was achieved. On the other hand, hydrogenated *Sterculia foetida* triglycerides contain 7.2% 8(9)-methylheptadecanoate and 46.9% 9(10)-methyloctadecanoate, which causes the poor resolution of triglyceride peaks (Figure 6). Therefore the present method for GLC analysis of odd carbon number triglycerides can be used only with straight-chain acids and not when a high percentage of branched-chain acids is present.

The presence of a cyclopentane ring in a triglyceride, however, makes that triglyceride elute later than its straight-chain analogue. In the *Hydnocarpus wightiana* chromatogram (Figure 6), hydrogenated trihydnocarpin elutes after hydrogenated palmitodihydnocarpin. Resolution between the two compounds is sufficient to form separate peaks, and a useful analytical separation results.

The calibration data in Table I and in previous communications (9, 11) indicate that significant amounts of C_{54} and higher triglycerides are lost during analysis. Although this does not affect accurate quantitation if proper calibration runs are made (9, 11), the question nevertheless arises about the cause of these losses. With on-column injection and a properly heated column, sample losses should occur in only two places: a) in the flash heater or b) on the column.

Flash heater losses could be caused by incomplete vaporization or pyrolysis. Comparison of the normal 350C flash heater injection with a cold injection procedure (i.e., the septum is removed and the sample placed directly in the glass wool on top of the packing) shows almost identical calibration values. Solvent fronts in the chromatograms are very narrow and clean; no peaks from low molecular weight pyrolysis products are observed. This holds true even when samples are dissolved in CS_2, to which the flame detector is very insensitive. On the other hand, with samples that contain triglycerides above C_{54}, small peaks are often present at a point where diglycerides produced from the sample might elute. Under optimum operating conditions these "diglyceride peaks" constitute less than 1% of total triglyceride peak area, and they have not been considered during quantitation. The mullet chromatogram in Figure 5 shows these minor "diglyceride peaks" eluting before carbon number 42. On the other hand, these "diglyceride peaks" increase markedly when Pyrex glass wool is placed in that part of the column heated by the flash heater.

Losses on the column could be attributable to irreversible adsorption, condensation, or pyrolysis at the high operating temperatures. The authors have no direct evidence that adsorption or condensation does or does not take place. Calibration chromatograms with triarachidin and tribehenin under the conditions of Table II do show a slight rise in the baseline immediately before the emergence of the main peak. This apparently indicates some on-column decomposition of C_{60} and C_{66} triglycerides at the high temperatures (above 350C) necessary for elution on a 1.83-m, 3.0% JXR column. However the amount of baseline rise before the triarachidin and tribehenin peaks is too little to account for more than 15% and 35% of the respective losses indicated by the calibration factors for these triglycerides in Table I.

In summary, the causes of sample losses

TABLE VII

Ratio of Odd Carbon Number Triglycerides to Odd Carbon Number Fatty Acids in Fish Oils

	Mullet A muscle	Mullet B body	Tuna muscle	Menhaden body	Pilchard body
% Odd carbon number triglycerides	37.3	31.2	10.9	8.6	7.3
% Straight chain odd carbon number fatty acids	20.4	16.7	4.2	3.0	2.4
Ratio of odd carbon number triglycerides to odd carbon number fatty acids	1.8	1.9	2.6	2.9	3.0

with triglycerides above C_{54} are still, for the most part, unknown. There is some evidence for a minor amount of flash heater pyrolysis, and some on-column degradation may occur with C_{60}-C_{66} molecules. But most of the lost sample is still unaccounted for, and one can only guess that it is adsorbed, condensed, or decomposed on the column.

Triglyceride Composition

All the fish oils analyzed show very wide carbon number distributions. Practically all possible di- and triacid triglycerides appear in the chromatograms, indicating a very wide distribution of fatty acids among the fish oil triglyceride molecules.

The ratio of total odd carbon number triglycerides to total odd carbon number straight-chain fatty acids is presented in Table VII. It is evident that the odd carbon number fatty acids are mostly distributed one per triglyceride molecule in tuna, menhaden, and pilchard oils, all of which contain less than 5% odd carbon number fatty acids. The two mullet samples have odd carbon number triglyceride: odd carbon number fatty acid ratios of 1.8 and 1.9, indicating that mullet oil contains many triglycerides having two odd carbon fatty acids per molecule.

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Search for New Industrial Oils. XIV. Seed Oils of Labiatae[1]

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ABSTRACT

Seed of 194 species in 56 genera of Labiatae, representing six of the eight subfamilies, were analyzed for oil and protein and for fatty acid composition of the oil. The oils are diverse and include some that contain up to 70% oleic acid, 79% linoleic acid, or 72% linolenic acid. An allenic function occurs in a third of the samples from the subfamily Stachyoideae and in the one sample analyzed from the Prasioideae. A method for determining the allene was devised. Oils from *Teucrium* species contain *trans* unsaturation in unidentified components. Oils from two *Lamium* species have both allenic and *trans* unsaturation. Two species of *Thymus* appear to produce hydroxy acids.

INTRODUCTION

THE LABIATAE (MINT FAMILY) consists of about 200 genera and 3,200 species. Though cosmopolitan in distribution, the family is particularly abundant in the Mediterranean region. The members of the family are predominantly annual or perennial herbs, sometimes shrubs, and rarely trees (1). Many secrete aromatic oils, and superficial oil glands are often visible on the leaves and flowers. These volatile aromatic essential oils account for a major share of the mint family's economic importance. According to Bailey (2), members of about 60 genera are cultivated in North America for odor, flavor, use in medicinal preparations, or as ornamentals. No species has been grown commercially in the United States as a source of glyceride oil, but, for some years prior to 1942, oil from *Perilla frutescens* seed was imported for use as a drying oil (3). In Russia some experimental work has been directed toward the production of *Elsholtzia* and *Lallemantia* oils (4).

When a search for new oils was begun in 1956, compositional data on seed oils from only 12 species of Labiatae were included in the major compilation (3,5). Even among those few the variability within the family was revealed by a wide range in content of the common unsaturated fatty acids. Early in the search, analyses of oils from 16 species (14 of which were newly reported) revealed the same compositional pattern and, within the limitations of the alkali-isomerization method, showed no unusual components (6-8). Following are results obtained by more definitive methods on 194 species of Labiatae, 12 of which were included in earlier work (6-8).

MATERIALS AND METHODS

Seed procurement, analysis of seed, and gas-liquid chromatography (GLC) of the methyl esters of the seed oils were essentially as described previously (6,9). For all species but one, nutlets (seed plus pericarp) were analyzed; the seed minus seed coat of *Salvia suffruticosa* was analyzed.

Preparation of Methyl Laballenate

Pure methyl laballenate (5,6-octadecadienoate) (10) was isolated by preparative GLC and thin-layer chromatography (TLC) of the mixed methyl esters from the seed oil of *Leonotis nepetaefolia*. The 18:2 peak in GLC, containing both laballenate and linoleate, was collected from an Aerograph Autoprep, Model A-700, equipped with a 20-ft × 3/8-in. copper column which was packed with 20% polyester LAC-2-R 446 on 60/80 mesh Celite. The column bath was at 200C, and helium flow was 600 ml/min. Laballenate was then separated from the other diene by TLC on a 1-mm film of Silica Gel G, containing 30% $AgNO_3$ on 10 × 34 cm plates. After the chromatogram was developed with benzene, the plates were sprayed with 0.2% dichlorofluorescein in 95% ethanol and observed under ultraviolet light. The bands containing methyl laballenate were removed by a vacuum device (11), and the ester was recovered by extraction with benzene. Neither GLC nor TLC revealed any impurity in the recovered product.

Determination of Laballenic Acid

A calibration curve for determining the

[1]Presented in part at the AOCS Meeting, Houston, April 1965.

[2]No. Utiliz. Res. Dev. Div., ARS, USDA.

[3]Crops Research Div., ARS, USDA.

allene was established by measuring the absorbance at 5.1 μ of six solutions of known concentrations of methyl laballenate in carbon disulfide (Fig. 1). One-millimeter KBr cells were used in a Perkin-Elmer infrared spectrophotometer, Model 337. Three additional standards were analyzed, in which methyl laballenate constituted 4.88, 9.80, and 19.5% by weight in admixture with methyl esters of myristic, palmitic, stearic, oleic, linoleic, and linolenic acids.

In determining laballenate, 75-330 mg of oil were weighed into a one-ml volumetric flask and made to volume with carbon disulfide. A one-mm KBr cell was filled with the solution and scanned across the region of the 5.1 μ band. The absorbance was determined as directed in AOCS Tentative Method Cd 14-61 (12) for *trans* isomers, and the concentration of laballenate was either read directly from the calibration curve or calculated on the basis of an average value of 0.104 for the absorptivity of methyl laballenate. Duplicate determinations were made on each oil that showed absorption at 5.1 μ.

For a few of the same oils, methyl esters were also analyzed by GLC on a 50-ft HI-PAK column (F&M Corporation), packed with 1% LAC-2-R 446. The laballenate peak was separated from others, and quantitative results were in good agreement with the IR method.

Oils from Lamium and Teucrium

For additional information on the unusual components in oils of *Lamium purpureum* and *Teucrium cubense* ssp. *laevigatum*, methyl esters from each oil were spotted on 20x20-cm TLC plates spread with a one-mm film of a mixture of 100 g Silica Gel G, 40 g $AgNO_3$, and 60 mg Rhodamine 6G. Chromatograms were developed with benzene in a 2-mm open "sandwich" chamber (13). Bands were observed and marked under ultraviolet light, then removed from the plate separately. Esters were recovered from the silica by extraction with ether, and each fraction was analyzed by IR for allene and *trans* unsaturation and by GLC on a 50-ft column.

RESULTS AND DISCUSSION

The 194 species of the Labiatae in this study (Table I) represent six of the eight subfamilies which were recognized by Briquet (14) in his classification system. Oils from this sampling of the family contain primarily the common fatty acids (Table I), but many also have an

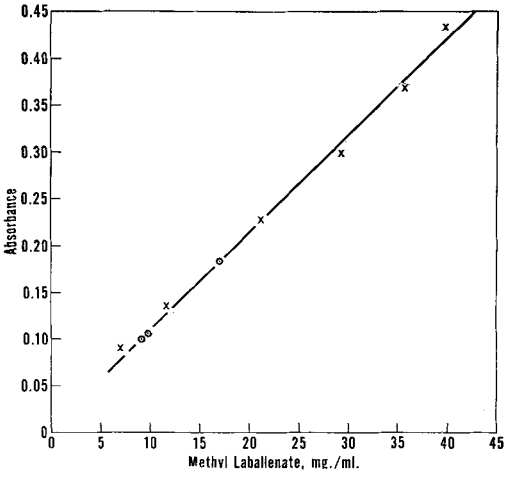

Fig. 1. Standard curve for determining methyl laballenate; ×, methyl laballenate; o, methyl laballenate in presence of other methyl esters. Cell length, 0.98 mm.

allenic component, presumably laballenic acid characterized in oil from *Leonotis nepetaefolia* (10), and several contain unidentified components. There are five oils with more than 75% linoleic acid, five with 65% or more oleic acid, and 12 with more than 65% linolenic acid. Oils from the subfamily Scutellarioideae (four species of *Scutellaria*) are all low in linolenic acid whereas those from the Lavanduloideae (five species of *Lavandula*) are all high. Oils from the Stachyoideae cover the full range of iodine value exhibited by the family, and those of the Ajugoideae extend over much of the mid-range (IV 110-186). Oils of the Ocimoideae are all in the high-IV region (IV 178-201) except that of *Hyptis suaveolens*, which differs distinctly in composition from other members of the subfamily. It is possible that the limited range in composition shown by subfamilies other than the Stachyoideae may expand as additional genera and species are examined.

With few exceptions the data are in reasonable agreement with data on species previously examined (3,15); differences in composition can probably be attributed to differences in sample origins or analytical methods. The largest discrepancies relate to oils from *Ocimum sanctum* and *Lallemantia royleana*, reported to have 66% linoleic acid and 60% oleic acid respectively (15). In contrast, oils of species reported here are both rich in linolenic acid. Oil from *Leonurus cardiaca*, reported to contain 35% 18:1 (6), is now shown to contain 21% 18:1 and 10% laballenic acid, a com-

TABLE I

Analytical Data on Labiatae Seeds and Oils

Source	Seed Analysis			Oil Properties				Composition of Methyl Esters, % (Area Percentage by GLC)						
	Wt/ 1,000 g	Oil, % DB	Protein, N x 6.25 % DB	Infrared analysis [1]	Refractive index n_D^{40}	Iodine value Wijs	Iodine value Calcd.	16:0	18:0	18:1	18.2[2]	18:3	Other Components Number	Other Components %
Ajugoideae														
Ajuga chia (Poir.) Schreb.	1.7	21	12	Usual	1.4705	147	149	5.8	2.4	22	58	11	3	0.1
Ajuga iva Schreb.	1.4	18	13	Usual	1.4691	144	144	7.2	3.1	24	53	12	5	0.4
Amethystea caerulea L.	0.6	23	16	Usual	1.4764	185	187	5.3	2.0	13	35	44	5	0.8
Rosmarinus officinals L.	0.8	12	14	FFA	1.4681	130	136	8.9	3.8	20	64	2.1	6	1.5
Tetraclea coulteri Gray	5.4	8.4	17	Usual	.....	110	109	9.2	3.5	48	32	3.9	10	3.5
Teucrium almeriense C. E. Hubb. and Sandw.	0.6	13	12	4.5% *trans*	1.4737	176	176	6.0	2.6	14	37	23	6	17
Teucrium capitatum L.	0.8	16	13	3.8% *trans*	1.4732	175	173	8.1	2.7	14	34	25	7	16
Teucrium chamaedrys L.	1.4	10	10	2.3% *trans*	1.4753	176	172	6.6	2.9	20	32	33	4	5.7
Teucrium creticum L.	3.0	8.4	7.8	5.4% *trans*	1.4700	142	143	6.7	1.8	30	43	0.4	9	18
Teucrium cubense Jacq. ssp. *laevigatum* (Vahl) McClint. and Epl.	2.7	15	11	5.9% *trans*	1.4704	140	142	7.8	4.0	26	45	1.0	6	17
Teucrium depressum Small	0.8	25	18	5.3% *trans*	1.4707	138	139	7.9	4.3	22	52	0.8	8	13
Teucrium expansum Pau	0.9	16	19	3.6% *trans*	1.4740	178	171	5.3	2.3	16	32	29	8	16
Teucrium flavum L.	1.6	11	10	2.5% *trans*	1.4733	166	165	6.9	3.0	22	27	34	7	7.0
Teucrium gnaphalodes Vahl	2.6	13	12	3.9% *trans*	1.4737	177	179	5.6	1.8	16	34	30	5	13
Teucrium polium L.	1.4	11	11	4.5% *trans*	1.4726	172	167	6.7	2.7	18	35	21	6	16
Teucrium pseudochamaepitus L.	7.5	10	14	2.9% *trans*	1.4684	125	124	8.8	4.0	37	41	0.6	5	8.7
Teucrium scordioides Schreb.	0.4	19	15	2.0% *trans*	1.4727	169	165	6.3	3.3	14	50	24	5	2.8
Teucrium scorodonia L.	0.4	13	13	2.9% *trans*	1.4746	186	178	6.1	3.5	18	27	39	3	6.8
Prasioideae														
Prasium majus L.	7.2	15	8.0	6.2% Allene [3]	1.4642	108	107	6.1	4.1	48	35	0.2	4	0.9
Scutellarioideae														
Scutellaria columnae All.	2.6	38	24	Usual	1.4670	119	123	7.8	2.7	33	54	0.1	5	2.4
Scutellaria condensata Rech. f.	0.8	16	....	.....	.....	.....	115	9.1	3.3	42	44	0.6	5	0.3
Scutellaria drummondii Benth.	0.5	33	22	Usual	1.4656	102	100	6.5	2.4	62	27	——	5	2.7
Scutellaria multicaulis Boiss.	0.5	42	28	Usual	1.4688	130	132	5.0	3.1	29	61	0.5	8	1.1
Lavanduloideae														
Lavandula dentata L.	0.2	31	24	Hydroxyl	1.4757	202	215	5.6	1.1	8.8	11	72	5	1.5
Lavandula lanata Boiss.	1.0	25	22	Usual	1.4760	198	207	5.5	2.1	10	16	65	7	1.5
Lavandula latifolia Medic.	1.1	28	23	Usual	1.4766	207	213	4.9	2.6	9.2	13	69	4	0.8
Lavandula pendunculata Cav.	0.1	20	27	Usual	1.4770	207	208	4.9	1.7	8.1	16	66	5	3.3
Lavandula stoechas L.	0.8	20	21	10.6 and 12.6 μ	1.4743	199	209	5.6	2.0	8.8	18	65	3	0.8
Stachyoideae														
Agastache urticifolia (Benth.) O. Ktze.	0.4	32	21	Usual	1.4745	186	196	4.0	1.3	16	24	55	3	0.9
Anisomeles indica (L.) O. Ktze.	1.2	28	15	Usual	1.4680	130	134	10	4.0	16	68	0.8	2	0.5

TABLE I (continued)

TABLE I (continued)

Analytical Data on Labiatae Seeds and Oils

Source	Seed Analysis			Oil Properties				Composition of Methyl Esters, % (Area Percentage by GLC)						
	Wt/ 1,000 g	Oil, % DB	Protein, N x 6.25 % DB	Infrared analysis [1]	Refractive index n_D^{40}	Iodine value Wijs	Iodine value Calcd.	16:0	18:0	18:1	18:2[2]	18:3	Other Components Number	Other Components %
Stachyoideae con't.														
Ballota acetabulosa Benth.	1.2	38	28	10% Allene	1.4675	114	114	7.0	3.6	34	43	0.8	4	0.8
Ballota hispanica (L.) Benth.	1.4	37	28	13% Allene	1.4676	112	111	7.7	3.2	32	41	0.8	6	1.6
Ballota nigra L. ssp. *ruderalis* (Sw.) Briq.	1.1	35	24	15% Allene[3]	1.4688	120	119	6.5	2.8	24	48	1.2	8	1.7
Betonica alopecuros L.	1.4	26	24	8.6% Allene	1.4689	127	129	5.7	3.0	26	55	1.7	6	0.3
Brazoria scutellarioides Engelm. and Gray	0.3	36	18	19% Allene	1.4707	134	136	6.7	2.0	30	14	27	6	1.6
Calamintha clinopodium Benth.	0.3	28	21	Usual	1.4765	205	206	4.6	2.6	7.3	25	60	6	0.7
Calamintha nepetoides Jord.	0.3	38	21	Usual	1.4758	196	204	4.5	2.3	13	20	61	4	0.2
Cleonia lusitanica L.	0.8	24	24	Usual	1.4756	195	205	5.4	2.3	15	9.7	67	6	1.1
Dracocephalum moldavica L.	1.5	25	21	Unident.	1.4747	198	209	5.6	2.6	7.3	16	67	6	1.8
Elsholtzia patrini Garcke	0.2	40	21	Unident.	1.4752	194	200	6.9	2.2	10	23	58	2	0.2
Elsholtzia splendens Nak.	0.3	39	21	Usual	1.4754	195	201	4.6	2.1	9.1	28	55	5	0.7
Eremostachys laciniata (L.) Bunge	25	12	21	11% Allene	1.4649	89	84	6.1	1.6	65	11	0.2	7	4.8
Eremostachys speciosa Rupr.	8.9	21	24	22% Allene	1.4667	87	81	5.6	2.2	53	8.8	0.9	9	7.7
Eremostachys vicaryi Benth.	6.3	19	25	15% Allene	1.4665	90	84	6.4	4.2	59	12	0.3	5	3.1
Hedeoma drummondii Benth.	0.2	6.9	8.8	Usual	1.4764	199	209	5.5	2.9	6.4	20	64	6	1.1
Hyssopus officinalis L.	1.0	29	27	11.5 μ	1.4758	197	202	5.4	2.6	12	17	62	7	1.1
Lallemantia canescens (L.) Fisch. and Mey.	3.7	32	20	Usual	1.4749	192	196	5.3	2.7	14	22	56	5	0.7
Lallemantia iberica (M.B.) Fisch. and Mey.	3.6	22	21	Usual	1.4758	200	201	6.9	2.2	14	9.4	66	6	2.2
Lallemantia royleana Benth.	1.6	20	18	Usual	1.4749	200	204	7.3	2.2	9.9	12	66	9	2.1
Lamium amplexicaule L.	0.6	39	23	12% Allene, 8.8% *trans*	1.4703	136	134	6.7	3.0	36	28[4]	11[5]	4	1.4
Lamium moschatum Mill.	7.0	22	13	5.4% Allene	1.4670	114	115	7.3	4.1	38	44	26	7	1.1
Lamium purpureum L.	1.0	39	22	14% Allene, 15% *trans*	1.4706	140	135	9.2	2.4	24	34[4]	12[5]	7	3.1
Leonotis nepetaefolia (L.) R. Br.	2.0	37	28	15% Allene	1.4662	92	87	11	5.5	47	19	0.6	5	2.5
Leonurus cardiaca L.	1.2	38	20	9.9% Allene	1.4694	130	131	4.6	2.3	21	55	3.6	8	3.1
Leonurus sibiricus L.	1.2	42	21	14% Allene	1.4693	124	121	4.9	1.8	24	50	1.0	7	4.8
Lepechinia spicata Willd.	1.4	16	12	Usual	1.4689	137	141	9.2	2.3	15	72	1.6	3	0.3
Leucas nutans Spreng.	2.6	43	19	16% Allene	1.4654	91	88	12	5.7	39	23	0.6	8	3.4
Lycopus asper Greene	0.7	25	17	Unident.	1.4719	155	185	3.5	1.5	16	24	50	5	5.4
Lycopus europaeus L.	0.6	33	19	11% Allene	1.4700	131	134	4.9	1.8	19	59	2.5	4	1.4
Marrubium peregrinum L.	1.0	32	26	10% Allene	1.4686	126	126	5.5	2.6	24	55	0.7	5	1.0
Marrubium pestalozzae Boiss.	1.1	34	24	8.6% Allene	1.4694	139	140	4.9	1.6	20	56	7.1	2	2.1
Marrubium vulgare L.	0.8	35	24	13% Allene	1.4682	118	116	7.3	2.0	31	44	1.1	2	1.0
Melissa officinalis L.	0.5	26	18	Usual	1.4759	201	206	5.1	2.1	6.0	29	58	3	0.2
Mentha aquatica L.	0.1	25	19	Usual	1.4750	184	194	5.7	2.8	11	29	51	6	0.4

Mentha arvensis L.	0.1	23	18	Usual	1.4752	199	207	5.2	2.2	9.5	21	62	1	Trace
Mentha longifolia (L.) Huds.	0.01	28	20	Usual	1.4762	197	203	5.0	2.1	7.3	30	56	3	0.4
Mentha pulegium L.	0.1	27	25	Usual	1.4754	193	198	6.4	4.5	6.5	25	57	5	0.9
Mentha rotundifolia (L.) Huds.	0.01	25	20	Usual	1.4750	193	195	6.3	2.8	8.5	30	52	5	0.6
Mentha sylvestris L.	0.1	25	19	Usual	1.4760	197	200	5.4	2.0	8.0	30	54	8	1.2
Mentha tomentosa D'Urv.	0.04	28	19	Usual	1.4753	191	197	4.8	2.7	11	29	52	6	0.6
Micromeria serpyllifolia (M.B.) Boiss.	0.7	28	20	Usual	1.4740	186	193	5.5	2.9	11	25	54	5	1.7
Moldavica parviflora (Nutt.) Britt.	1.6	18	15	Usual	1.4744	187	189	6.5	2.9	11	29	50	4	1.0
Molucella laevis L.	4.5	34	24	8.9% Allene	1.4663	102	100	5.6	2.8	55	26	0.4	5	1.0
Molucella spinosa L.	5.9	38	20	10% Allene	1.4658	103	107	7.1	1.5	47	33	0.5	4	1.3
Monarda fistulosa L. var. *menthaefolia* (Graham) Fern.	0.5	36	28	Usual	1.4770	208	220	2.8	1.6	8.7	17	70	—	——
Monarda punctata L.	0.2	31	21	12.6 μ	1.4769	208	203	4.6	4.9	7.7	18	65	3	1.5
Monardella sp.	0.6	27	19	2.8% Allene[3]	.....	.....	204	5.4	2.6	8.8	17	63	4	0.5
Mosla punctulata (J. F. Gmel.) Nakai	0.2	29	22	Usual	1.4759	201	207	6.4	2.3	8.5	17	65	3	0.1
Nepeta cataria L.	0.5	21	18	1.2% Allene[3]	1.4754	201	201	6.0	2.0	10	20	60	3	1.3
Nepeta congesta Fisch. and Mey.	1.0	17	18	Usual	1.4766	202	207	5.4	1.6	10	19	64	3	0.2
Nepeta glomerulosa Boiss.	0.3	20	22	Usual	1.4753	195	199	7.8	7.7	11	17	61	8	0.3
Nepeta italica L.	0.3	22	17	Usual	1.4757	196	203	4.9	2.3	10	23	59	6	0.7
Nepeta latifolia DC.	0.8	17	22	Usual	1.4756	196	204	5.2	1.6	11	20	61	5	0.9
Nepeta mussinii Spreng.	0.8	25	22	Usual	1.4760	200	206	4.7	2.2	9.2	21	61	7	1.2
Nepeta nepetella L.	0.4	27	19	Usual	1.4754	194	198	6.6	2.8	10	22	58	6	0.7
Nepeta nuda L. var. *albiflora* Boiss.	0.5	23	22	Usual	1.4752	197	203	5.3	2.5	11	18	62	7	1.1
Nepeta pannonica L. var. *pannonica*	0.4	22	24	11.5 and 12.6 μ	1.4754	197	199	5.0	2.0	11	22	58	10	2.7
Nepeta spicata Benth.	0.4	41	22	Usual	1.4757	202	206	5.0	3.2	11	17	64	2	0.1
Nepeta tmolea Boiss.	0.6	29	22	Usual	1.4761	200	206	5.3	3.0	8.5	18	64	6	1.0
Nepeta tuberosa L.	0.5	19	25	FFA	1.4764	202	206	5.6	2.1	8.7	13	68	8	3.4
Origanum vulgare L.	0.1	35	26	Usual	1.4772	210	216	4.5	1.9	5.4	22	66	2	0.2
Otostegia limbata (Benth.) Boiss.	4.5	43	34	16% Allene	1.4652	88	84	11	4.8	53	14	0.3	5	1.6
Perilla frutescens (L.) Britt.	1.4	35	20	Usual	1.4741	187	192	6.6	2.0	15	19	56	4	1.2
Phlomis armeniaca Willd.	7.7	26	36	8.9% Allene[3]	1.4650	95	95	4.1	0.9	68	16	0.7	6	1.2
Phlomis austra-anatolica Hub. Mur.	6.4	14	24	20% Allene	1.4662	85	82	7.0	1.9	61	7.4	0.3	6	2.0
Phlomis crinita Cav.	11	24	33	12% Allene	1.4646	88	82	7.7	1.7	64	10	0.1	10	4.3
Phlomis fruticosa L.	10	18	30	12% Allene	1.4646	86	85	7.3	2.4	68	8.5	0.8	7	1.2
Phlomis herba-venti L.	10	30	35	5.6% Allene	1.4658	104	105	4.8	1.4	58	28	0.8	4	1.9
Phllomis lycia D. Don	6.0	18	32	13% Allene	1.4646	84	82	6.3	1.6	70	6.5	0.1	6	1.3
Phlomis purpurea L.	4.4	21	33	15% Allene	1.4647	82	81	7.6	3.4	66	6.8	Trace	7	1.1
Phlomis rigida Labill.	15	16	36	12% Allene	1.4657	92	89	7.4	1.5	62	14	0.5	8	2.3
Physostegia virginiana (L.) Benth.	2.6	35	25	12% Allene[3]	1.4705	.....	146	3.9	2.0	29	31	22	3	0.7
Pogostemon parviflorus Benth.	0.2	36	25	Usual	1.4685	139	143	8.5	3.3	11	76	1.0	3	0.7
Prunella asiatica Nak.	0.8	20	19	Usual	1.4754	194	200	5.7	2.3	13	19	59	4	0.7
Prunella vulgaris L.	0.9	19	19	12.6 μ	1.4742	189	196	7.7	2.1	14	16	60	2	0.5
Prunella vulgaris L. var. *lanceolata* (Bart.) Fern	1.8	22	17	Usual	1.4740	193	194	8.4	2.1	13	17	59	1	0.7
Pycnanthemum muticum Pers.	0.4	35	31	Usual	1.4772	209	212	2.9	2.8	9.2	19	65	6	1.2
Salvia acetabulosa L. var. *simplicifolia* Boiss.	6.0	18	23	2.3% Allene[3]	1.4686	132	138	9.1	1.5	19	67	1.3	6	0.5
Salvia aegyptiaca L.	0.6	22	20	Usual	1.4746	190	194	8.0	3.5	11	21	57	2	0.2
Salvia aethiopis L.	2.2	22	22	Usual	1.4747	186	192	5.8	2.4	20	12	58	6	1.0
Salvia amplexicaulis Lam.	0.9	28	23	Usual	1.4738	181	185	6.2	3.2	11	36	44	4	0.6
Salvia apiana Jeps.	1.2	6.9	7.9	.....	1.4733	160	168	7.1	3.4	21	31	36	8	1.0
Salvia bicolor Desf.	7.0	24	22	Usual	1.4666	119	123	14	2.6	25	55	2.1	7	1.5

TABLE I (continued)

TABLE I (continued)

Analytical Data on Labiatae Seeds and Oils

Source	Seed Analysis			Oil Properties				Composition of Methyl Esters, % (Area Percentage by GLC)						
	Wt/ 1,000 g	Oil, % DB	Protein, N x 6.25 % DB	Infrared analysis [1]	Refractive index n_D^{40}	Iodine value Wijs	Iodine value Calcd.	16:0	18:0	18:1	18:2[2]	18:3	Other Components Number	Other Components %
Stachyoideae con't.														
Salvia brachyantha (Bordz.) Pobedim.	0.9	22	23	Usual	1.4733	176	182	8.1	1.8	14	29	45	4	1.5
Salvia bracteata Russ.	10	20	19	Usual	1.4683	132	134	8.0	2.6	24	64	0.5	5	0.8
Salvia carduacea Benth.	2.2	30	26	Usual	1.4698	148	149	9.3	2.8	34	19	32	7	2.2
Salvia ceratophylla L.	9.6	19	17	Usual	1.4740	179	185	5.0	1.6	14	36	42	1	1.0
Salvia coccinea Juss. ex Murr.	1.0	24	16	FFA	1.4706	158	164	8.5	7.1	12	38	34	4	0.7
Salvia columbariae Benth.	0.5	32	24	Usual	1.4758	202	208	5.8	2.8	8.9	17	65	4	0.6
Salvia cryptantha Montbr. and Auch.	6.1	11	17	Usual	1.4690	135	142	5.4	2.3	20	71	0.7	6	0.7
Salvia euphratica Montbr. and Auch.	34	21	18	Usual	1.4687	138	140	6.8	2.0	21	67	1.6	5	0.9
Salvia farinacea Benth.	1.2	17	15	Usual	1.4726	184	186	7.6	2.6	17	22	51	1	0.2
Salvia glutinosa L.	4.0	39	24	Usual	1.4730	176	178	5.2	2.6	15	39	37	5	1.1
Salvia grandiflora Etling.	6.0	16	17	Usual	1.4677	124	126	11	2.6	26	58	0.7	6	1.2
Salvia hispanica L.	0.4	29	18	Usual	1.4753	196	201	7.1	3.3	6.8	24	59	3	0.2
Salvia horminum L.	2.7	24	22	Usual	1.4725	173	172	9.0	2.6	19	27	41	6	1.1
Salvia hydrangea DC. ex Benth.	4.1	16	20	Usual	1.4672	122	123	10	2.4	32	53	0.9	6	0.9
Salvia judaica Boiss.	0.6	20	19	Usual	1.4754	192	195	6.0	2.8	7.7	30	52	3	1.8
Salvia lanigera Poir.	1.0	23	19	11.6 and 12.6 μ	1.4735	182	184	8.7	2.0	16	21	51	6	1.4
Salvia lavandulaefolia Vahl	3.0	21	19	Usual	1.4685	137	141	7.9	2.2	18	69	2.1	5	0.9
Salvia longispicata Mart. and Gal.	0.2	19	19	Usual	1.4745	188	189	7.1	4.9	8.7	26	52	7	1.3
Salvia lyrata L.	1.2	23	21	Usual	1.4679	133	138	8.6	3.5	17	70	1.0	4	0.5
Salvia mexicana L.	2.4	14	16	Usual	1.4740	187	192	5.9	3.9	11	26	52	6	0.6
Salvia montbretii Benth.	10	21	19	Usual	1.4703	153	159	7.5	2.7	14	55	20	6	0.8
Salvia moorcraftiana Wall. ex Benth.	3.3	27	23	Usual	1.4705	154	159	9.6	3.4	27	23	36	3	0.3
Salvia nemorosa L.	1.1	27	26	Usual	1.4750	193	199	4.8	1.9	9.2	30	53	5	0.8
Salvia officinalis L.	8.6	25	22	Usual	1.4696	143	146	7.2	2.4	13	76	0.9	4	0.5
Salvia plebeia R. Br.	0.2	21	18	Unident.	1.4758	159	163	8.0	2.9	13	37	33	5	5.2
Salvia polystachya Ort.	0.2	33	23	Usual	1.4750	190	196	6.1	3.2	11	21	57	6	1.3
Salvia pratensis L.	1.0	19	24	FFA	1.4748	185	194	5.9	2.6	9.3	31	50	4	0.6
Salvia reflexa Hornem.	1.4	23	19	Usual	1.4760	199	202	6.6	3.0	11	16	63	2	Trace
Salvia ringens Sibth. and Sm.	4.7	13	19	FFA	1.4688	131	134	7.5	2.4	21	65	0.8	9	2.5
Salvia rosaefolia Sm.	4.4	20	20	Usual	1.4685	136	139	6.9	2.4	21	68	1.0	3	0.8
Salvia rugosa Dryand. ex Ait.	2.4	28	19	Usual	1.4716	165	172	8.2	3.6	17	32	39	4	0.4
Salvia sclarea L.	3.0	31	28	11.6 μ	1.4729	185	189	6.9	2.5	18	17	54	4	0.7
Salvia similata Haussk.	6.1	11	17	Usual	1.4730	163	171	7.6	2.9	21	27	40	7	1.3
Salvia sonomensis Greene	1.0	21	16	Usual	1.4718	162	168	5.4	2.7	21	37	32	7	1.3
Salvia suffruticosa Montbr. and Auch.[6]	15	40	26	Usual	1.4679	132	137	8.4	1.7	21	67	0.7	5	0.9
Salvia sylvestris L.	1.0	27	23	Usual	1.4750	192	198	4.2	2.3	14	24	55	3	0.2
Salvia syriaca L.	1.4	12	16	Usual	1.4735	174	182	5.0	2.2	17	31	43	7	1.6
Salvia tchihatcheffii (Fisch. and Mey.) Boiss.	4.5	22	20	Usual	1.4705	150	151	4.8	1.6	13	79	12	3	0.2
Salvia texana (Sheele) Torr.	2.0	16	17	Usual	1.4690	140	141	11	2.9	8.9	76	12	2	0.2
Salvia triloba L. f.	7.3	23	19	Usual	1.4667	124	126	9.2	5.2	25	59	0.8	4	0.6
Salvia valentina Vahl	1.0	14	24	FFA	1.4732	176	182	8.4	2.9	12	31	45	3	0.2
Salvia verbenaca L.	0.6	18	19	Usual	1.4736	177	180	11	2.1	14	25	48	4	0.5

Salvia verticillata L.	0.5	19	18	FFA	1.4723	165	168	6.6	3.2	20	34	35	7	1.2
Satureja coreana (Levl.) Nakai	0.1	26	16	Usual	1.4754	197	200	4.7	2.7	9.0	28	54	6	0.7
Satureja hortensis L.	0.6	39	23	Usual	1.4765	207	212	4.3	1.7	7.6	20	65	5	1.4
Satureja thymbra L.	0.6	18	13	Usual	1.4745	185	188	5.3	2.2	18	26	49	4	0.2
Sideritis hirsuta L.	1.4	35	16	9.3% Allene	1.4691	131	133	4.7	2.4	22	61	0.4	4	0.1
Sideritis incana Benth.	1.6	29	17	13% Allene	1.4693	127	127	4.3	1.8	23	55	0.6	3	2.2
Sideritis lagascana Willk.	1.4	36	20	10% Allene	1.4690	134	132	5.0	3.6	18	60	2.0	5	1.3
Sideritis leucantha Cav.	1.0	34	14	11% Allene	1.4694	129	131	5.6	3.0	19	58	0.6	5	1.3
Sideritis linearifolia Lam.	1.0	37	19	10% Allene	1.4694	132	133	3.9	1.7	21	61	1.8	4	1.3
Sideritis montana L. var. *comosa* Boiss.	0.8	38	19	8.7% Allene	1.4707	139	142	3.4	1.8	19	66	1.5	—	——
Sideritis taurica Willd.	1.1	36	23	4.6% Allene	1.4693	136	137	2.6	1.0	28	62	0.8	4	0.6
Sideritis tragoriganum Lag.	1.2	28	15	13% Allene	1.4694	128	128	6.0	2.6	19	56	1.4	4	1.4
Stachys arvensis L.	0.8	38	15	12% Allene[3]	1.4693	130	126	5.8	3.3	20	52	3.2	4	3.0
Stachys cretica Sibth. and Sm.	3.6	29	16	3.9% Allene	1.4688	135	131	4.8	2.1	29	59	0.1	4	1.3
Stachys germanica L.	1.3	30	17	4.7% Allene	1.4698	140	140	4.2	2.0	19	68	0.8	4	0.4
Stachys hirta L.	0.6	32	14	11% Allene	1.4681	124	126	6.5	3.0	21	55	1.5	6	1.6
Stachys milanii Petrov.	1.8	32	14	6.8% Allene	1.4701	139	140	2.0	0.4	28	54	8.2	5	0.9
Stachys olympica Poir.	2.4	32	20	3.8% Allene	1.4686	144	139	4.8	3.0	15	71	——	2	2.2
Stachys sylvatica L.	0.8	22	14	6.2% Allene	1.4696	128	132	6.3	2.8	23	52	6.6	5	2.4
Stachys thirkei C. Koch	1.5	29	18	4.4% Allene	1.4692	137	138	4.2	3.3	20	67	0.4	3	1.2
Stachys viticina Boiss.	1.3	31	14	5.5% Allene	1.4682	126	127	5.8	3.1	27	57	0.4	4	1.0
Thymbra spicata L.	3.4	6.4	10	Hydroxyl	1.4740	186	193	7.2	2.5	12	22	55	3	0.9
Thymus capitatus (L.) Hoffm. and Link	0.3	37	33	Hydroxyl, 6.3 and 11. 5 μ	1.4761	193	191	6.4	3.0	12	21	55	5	2.3
Thymus chaubardii Boiss. and Heldr. ex Boiss.	0.3	38	34	Usual	1.4823	191	188	5.3	2.8	13	17	56	6	6.2
Thymus vulgaris L.	0.6	37	28	Hydroxyl	1.4783	204	——	1.6	1.8	7.0	14	54	7	21
Thymus zygis L.	0.2	30	26	Hydroxyl, 7.3 and 11.5 μ	1.4783	206	——	4.6	2.4	2.6	14	56	7	14
Wiedemannia orientalis Fisch. and Mey.	1.2	26	40	7.0% Allene	1.4706	142	135	5.1	2.5	23	50	8.7	6	2.4
Ziziphora capitata L.	0.4	34	26	Usual	1.4768	209	216	5.9	2.1	6.5	14	71	2	0.4
Ziziphora tenuior L.	0.4	32	25	Usual	1.4756	207	215	5.2	2.0	7.0	14	70	5	1.3
Ocimoideae														
Hyptis decurrens (Blanco) Epling	0.5	20	15	Usual	1.4740	189	196	5.6	3.2	8.2	31	51	2	0.3
Hyptis floribunda Briq.	0.4	25	17	Usual	1.4749	185	195	6.5	2.5	12	24	55	3	Trace
Hyptis mutabilis (Rich.) Briq.	0.1	30	22	Usual	1.4757	196	201	6.8	2.7	8.3	23	58	4	0.6
Hyptis spicigera Lam.	0.6	19	14	Usual	1.4757	201	212	5.4	2.1	6.1	22	64	1	0.1
Hyptis stellulata Benth.	0.3	22	20	Usual	1.4750	191	201	7.2	2.7	7.7	24	58	2	Trace
Hyptis suaveolens (L.) Poit.	7.4	24	22	Usual	1.4696	145	146	8.1	2.9	8.5	79	0.6	3	0.9
Ocimum americanum L.	0.9	18	18	Usual	1.4753	186	194	6.2	3.1	15	17	58	6	0.9
Ocimum basilicum L.	1.3	24	21	Usual	1.4746	191	196	7.4	2.9	9.2	25	56	4	0.3
Ocimum sanctum L.	0.9	16	14	Usual	1.4748	192	196	6.9	2.6	7.5	29	54	6	0.7
Ocimum selloi Benth.	0.6	28	21	Usual	1.4731	178	184	7.3	2.9	12	33	44	3	0.2
Plectranthus inflexus (Thunb.) Vahl ex Benth.	0.8	39	25	Usual	1.4754	201	204	4.5	2.1	10	25	58	1	0.1

[1] *Trans* acids calculated as elaidate, allenic acids as laballenate.

[2] Total 18:2 minus laballenate except as noted.

[3] Determined on the methyl esters.

[4] Total 18:2.

[5] Total 18:3 minus allene (calculated as laballenate).

[6] Seed minus seed coat analyzed.

ponent not revealed by the alkali-isomerization method. Similarly the isomerization method revealed nothing unusual in oil of *Thymus vulgaris* and barely suggested unusual composition in oil of *Lycopus asper* by indicating a complete absence of saturated acids.

The general pattern of composition of the oils is revealed by plotting fatty acid content against iodine value (Fig. 2). The percentage of 18:2, assumed to be mostly linoleic acid, is shown for each oil, but the amounts of 18:1 (probably oleic acid) and 18:3 (probably linolenic acid) are indicated only as general regions. The oils appear to fall into two groups. In the low range of iodine value, increasing iodine values result from increasing amounts of linoleic acid and a corresponding decrease in oleic acid. Linolenic acid is absent or increases only slightly. Similar relationships have been recorded for cottonseed (16) and soybean oils (17). In the high range of iodine value, linolenic acid increases whereas both linoleic and oleic acids decrease. This pattern is similar to that in linseed oil (18,19). The two patterns overlap in the IV range from about 130-150.

Oils Containing Laballenic Acid

Laballenic acid was found only in the subfamily Stachyoideae in 52 of 156 species, and in the single representative of the Prasioideae. It occurs primarily in oils that have little or no linolenic acid. Only three of the oils have IV above 145 (*Monardella* sp., *Nepeta cataria*, and *Physostegia virginiana*), and only three others have as much as 10% 18:3 (*Brazoria scutellarioides* and two *Lamium* species). The two *Lamium* species have the further distinction of producing an allenic acid of novel structure (vide infra). The occurrence of an allene in only one of the 49 species of *Salvia* is surprising; the allenic component has not been identified.

In determining laballenate in oils by the infrared method, the mean difference between duplicates is 0.20%. Results on two oils were somewhat lower than measurements made on methyl esters from the oils (15.1 vs. 15.9% and 13.7 vs. 14.0%). It is possible that separate standard curves should be prepared for analysis of oils, free acids, and methyl esters, as is required in the determination of *trans* unsaturation by AOCS Tentative Method Cd 14-61 (12). Data for allenes in Table I were obtained from either oils or esters as governed by availability of sample, and no effort was made to correct for the application of the ester calibration to analysis of oils.

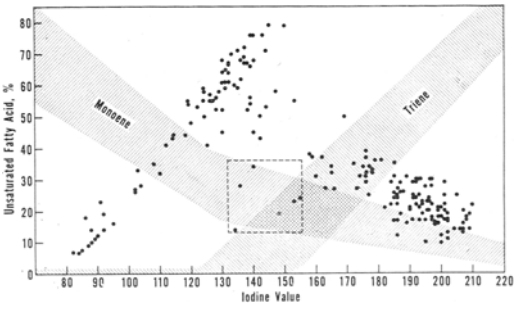

Fig. 2. Composition of Labiatae oils as related to iodine value. Individual samples indicated for diene only. Regions indicated for monoenes and trienes.

Oils That Contain Unidentified Components

Absorption of IR radiation by oils from *Lamium amplexicaule* and *L. purpureum* indicates the presence of *trans* unsaturation and an allenic function. Preparative TLC of the methyl esters of *L. purpureum* on Silica G-$AgNO_3$ results in five fractions, the first two of which contain all the allene and the second of which includes almost all the *trans* bonds. Band No. 1 was in the area normal for saturated esters and methyl laballenate. Analysis by IR showed 13.2% laballenate, equivalent to 1.7% on the basis of the total methyl esters of the oil. GLC of this fraction indicated 10.6% of laballenate. Band No. 2 was in the monoene region, and GLC indicated 64% 18:1 and 32% of an unidentified component. IR shows the presence of 36% allene (calculated as methyl laballenate) and 34% *trans* ester (calculated as methyl elaidate). The unknown component apparently contains both the allenic structure and the *trans* bond. TLC supports this conclusion in part because the unknown migrates with the monoenes and should have one double bond more than methyl laballenate, which migrates with the saturated esters. GLC provides similar support because the retention time of the unknown is near that of 18:3 whereas that for methyl laballenate is near 18:2. From the IR analysis of Band No. 2 the original oil has 13.3% of the unknown. Chemical characterization of the unknown will be reported elsewhere (20). Band No. 3, in the region of normal dienes, contains enough *trans* unsaturation to represent 2% of methyl elaidate in the mixed esters, but the nature of the component has not been determined.

Oils of the genus *Teucrium* contain *trans* unsaturation although the amount in two of the oils was so low it escaped detection in the qualitative IR examination used. In TLC on Silica

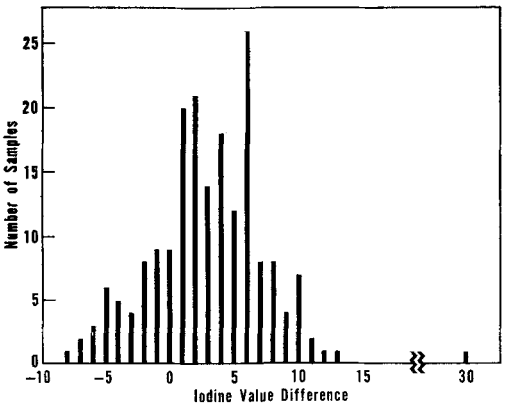

Fig. 3. Difference between the iodine value calculated from GLC analysis and that determined by Wijs method.

G-$AgNO_3$ all the *trans* unsaturation migrates with the usual trienes. The "triene" band tends to divide, and GLC of the two portions indicates the presence of linolenic acid and four other components, one of which has GLC retention characteristics of a C_{18} diene.

Methyl esters from the oils of *Thymus vulgaris* and *T. zygis* contain 21 and 14% respectively of an unidentified component which has equivalent chain-lengths (ECL) of 18.7 (Apiezon L) and 23.8 (LAC-2-R 446). *T. chaubardii* oil has about 2% of the same material. The ECL's suggest that the acid moiety is a C_{17} hydroxy acid. The presence of the hydroxyl group is confirmed by IR absorption of the esters.

Further Indications of Unusual Composition

The plot of linoleic acid against IV (Fig. 2) shows six oils (in box) between IV 134 and 155 with less than 35% linoleic acid, distinctly less than the other oils of comparable IV. Two of the six are *Lamium* species, and the deviation from the general pattern of the family may be related to the presence of the unknown allene mentioned above. Oil from *Brazoria scutellarioides* contains an allene but differs from the two *Lamium* oils in that it has no *trans* unsaturation. Two oils are from *Salvia carduacea* and *S. moorcroftiana,* and no explanation for their deviation from the pattern is apparent at present. The sixth oil, from *Lycopus asper,* shows numerous weak absorption bands in the IR and several small unidentified components by GLC.

Further evidence of unusual components in Labiatae oils, especially oil of *L. asper,* is given by the distribution pattern of the differences between the measured (Wijs) IV and that calculated from the GLC analyses (Fig. 3). Differences ranged from –8 to +13 except for *L. asper* oil, which gave +30. This range is somewhat greater than that encountered, but not published, in work on the Boraginaceae (21), Euphorbiaceae (22), and Cruciferae (23). Further the Labiatae histogram peaks at about +2, as do the three other families, but the number of samples at +6 and above suggests the presence of components which give unusual response in the GLC or IV analyses.

In calculating the IV differences, an empirical value was used for the IV of laballenate. Since conjugated unsaturation adds less than the stoichiometric amount of Wijs reagent, the allenic unsaturation was expected to give a similar incomplete reaction. Reaction of Wijs reagent with methyl laballenate for 30 min resulted in an IV of 81.3 whereas complete saturation of one double bond would have given 86.2 and saturation of both would have given 172.4. Use of a small sample, 0.1 g, and the incomplete reaction resulted in an excess of reagent of about 550%. If the excess reagent were held to the usual 100-150%, the observed IV should be lower but still might not represent the situation in the determination of IV of an oil because of the more rapid reaction of the common unsaturated acids with the reagent. Peer (24) has shown that the reaction of bromine in glacial acetic acid with the allene of propadiene is about half complete at 1½ hr and still incomplete at 72 hr. The reaction of bromine in chloroform is also slow, but at a given time the extent of the reaction is increased by increased concentration of the reagent. The progress of the reaction with the Wijs reagent is similar to that of bromine in acetic acid (24), and it seems probable that the effect of reagent concentration would also be like that with bromine.

For those oils containing allene, the IV differences range from –7 to +6 and the mode is +1. A preponderance of negative differences supports the suggestion that the reaction of Wijs reagent with allene in the presence of other unsaturated acids may be less than that measured with the large excess of reagent in the test cited above.

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Fatty Acid Concentrations in Synchronous Cultures of *Chlorella pyrenoidosa*, Grown in the Presence and Absence of Glucose

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ABSTRACT

Total cellular fatty acids, of a high-temperature strain of *Chlorella pyrenoidosa*, were measured during a synchronous growth cycle in continuous light in the presence and absence of glucose.

The fatty acid composition, consisting of saturated monoenoic, dienoic, and trienoic C_{16} and C_{18} fatty acids, remained essentially the same whether or not the cells were grown on glucose.

Nearly all of the fatty acids increased irregularly in concentration during the growth cycle, showing a periodism during the prenuclear and cell-division stages of growth. Cultures exposed to glucose showed a more pronounced drop in the concentration of most of the fatty acids during both stages.

None of the fatty acid concentrations, some of which have been implicated in the Hill reaction of a number of photosynthetic organisms, reflected the periodism in photosynthetic activity which was previously observed during the synchronous growth cycle of this organism.

The changes in fatty acid concentration are discussed with relation to concurrent metabolic and cytological changes during cell maturation in this organism.

INTRODUCTION

THE FATTY ACID COMPOSITION of algae has been investigated under a variety of nutritional and physiological conditions (1,2). These data however have been obtained from cell cultures which contain cells of all ages. Though the general fatty acid pattern was established, little, if any, information has been gathered relating changes in either the pattern or the concentrations of these lipids to the process of cell maturation.

Problems concerning the growth and maturation of cells have been successfully approached by using cell populations the member cells of which all have been induced to grow and divide more or less simultaneously (3,4). In such "synchronized cultures" visual cytological changes (5) (mitosis, cross-wall deposition, cytokinesis) occur more or less in phase and can be related to the metabolic changes (measured chemically) which accompany them (3).

Chlorella pyrenoidosa, a unicellular alga, can be induced to grow synchronously in mass cultures; at the same time it exhibits relatively normal logarithmic growth in other respects (6-8).

The cellular demands for energy- and carbon-building blocks during the nuclear division and cross-wall deposition stages of growth, coupled with an increase in the cellular respiration rate (9) and a decline in the photosynthetic activity (10) during the same period in synchronized *Chlorella* cultures, have led Duynstee and Schmidt (11) to infer that carbohydrate and/or lipid reserves which accumulate prior to this growth period might be mobilized at the time to meet the metabolic demands of these processes.

The α-linolenic acid (12) as well as *trans* 3-hexadecenoic acid (13) have been suggested as being involved in the Hill reaction in various photosynthetic organisms. The absence of both of these fatty acids in two species of blue-green algae, which were nevertheless capable of performing the Hill reaction, has led Nichols (14) to conclude that the essential participation of either α-linolenic acid or *trans* 3-hexadecenoic acid in the Hill reaction can be discounted. The lack of universality in the distribution of these fatty acids does not however automatically preclude the possibility of their intimate involvement in the photosynthetic processes of green algae and higher plants. Synchronized cultures of the green

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algae *Chlorella pyrenoidosa* might afford a convenient test of this involvement by virtue of a dramatic periodism in the photosynthetic activity (10) during its growth cycle. Fatty acids associated with the photosynthetic process might be expected to exhibit a related periodism in their concentrations during a synchronous growth cycle of this organism.

The purpose of the present investigation was to determine whether possible periodism in the cellular levels of the fatty acids would implicate their involvement in the photosynthetic process and in the process of supplying carbon-building blocks and/or energy reserves at select intervals during the growth cycle of *C. pyrenoidosa.*

MATERIALS AND METHODS

Organism

The alga used in these studies was the high-temperature strain 7-11-05 of *C. pyrenoidosa,* described by Sorokin (15). The cells were synchronized by using the intermittent illumination method described by Schmidt (6).

Culture Conditions

The cells were grown in 2 × 21-in. bubbler tubes immersed in a constant temperature (38.5C) water bath and illuminated by two banks of 40-watt fluorescent lights (eight each), which were mounted 3 in. from the outside edge of the tubes. The liquid medium, described by Sorokin (15) and modified by Johnson (16), was aerated with a CO_2-air mixture (3%). The entire growth chamber was housed in a light-tight cabinet.

Synchronized cultures grown for one growth cycle in continuous light under the above conditions exhibited a linear logarithmic increase in dry weight and phosphorus identical to that reported in other laboratories for this organism (6) (see Results).

The fatty acid content of the cells was measured at intervals during a synchronous growth cycle of *C. pyrenoidosa* in the presence of light: a) normal mineral medium (light, nonglucose)-(photo-autotrophic); b) normal mineral medium plus 0.2% glucose (light, glucose)-(photoheterotrophic). The conditions of temperature and aeration were identical for both experiments. Where indicated, sufficient sterile glucose, autoclaved separately from the mineral medium, was added to give a final concentration of 0.2% w/v.

Biochemical Measurements

Cellular dry weight (17) and total cellular phosphorus (18) were determined in triplicate. Cell number per ml was determined by using a Levy-Hauser hemocytometer.

The samples for triplicate lipid analysis were harvested directly from the culture tubes and placed into a Servall refrigerated centrifuge. The cells were centrifuged at 27,000 × *g* for 7 min. and washed three times with an ice-cold solution of 0.125% (w/v), Tris (Tris hydroxyamino methane) 0.01% $CaCl_2$ (w/v), pH 7.0, to remove residual media materials. After the final wash about 5 ml of cold distilled water were used quantitatively to transfer the cell material into Teflon-lined screw-cap test tubes. Five ml of cold absolute ethanol were added, and the sealed tubes were stored in a darkened refrigerator overnight.

Each sample was then sonicated by using the micro-tip attachment of a Branson Sonifier (Model 125), which was operated at a maximum power for 5 min. The sonifier probe was immersed deeply into each sample tube, and the tube was cooled in a circulating acetone-ice bath at −18C. During the five minutes of sonication the contents of the tube remained below 5C.

A known amount of myristic acid, a fatty acid which had been shown to be present in only trace quantities during a previous synchronous growth cycle, was added as an internal standard. Enough aqueous KOH (100 g/100 ml) to make the final solution 10% KOH was added. The tubes were tightly sealed and placed in a water bath at 60C for one hour. The sealed tubes were allowed to stand overnight in the dark at room temperature, and the tube contents were again heated for one hour in a water bath, but this time at 70C. Upon cooling, the cell material was extracted three times with n-hexane to remove the nonsaponifiable material. The mixture was then acidified with 25% HCl and extracted three more times with n-hexane. This extract was washed free of HCl with water and evaporated under nitrogen at 45C. The methyl esters were made by adding two drops of methanol to the extract, then 0.5 ml of an ethereal solution of diazomethane. This mixture was allowed to sit approximately one minute, and the excess ether and diazomethane were evaporated under nitrogen at 45C. Then CS_2 was added to the residue, and the methyl-esters were analyzed by gas-liquid chromatography (GLC).

Chromatographic Analysis

Gas chromatographic analysis of fatty acid methyl esters was performed with a gas

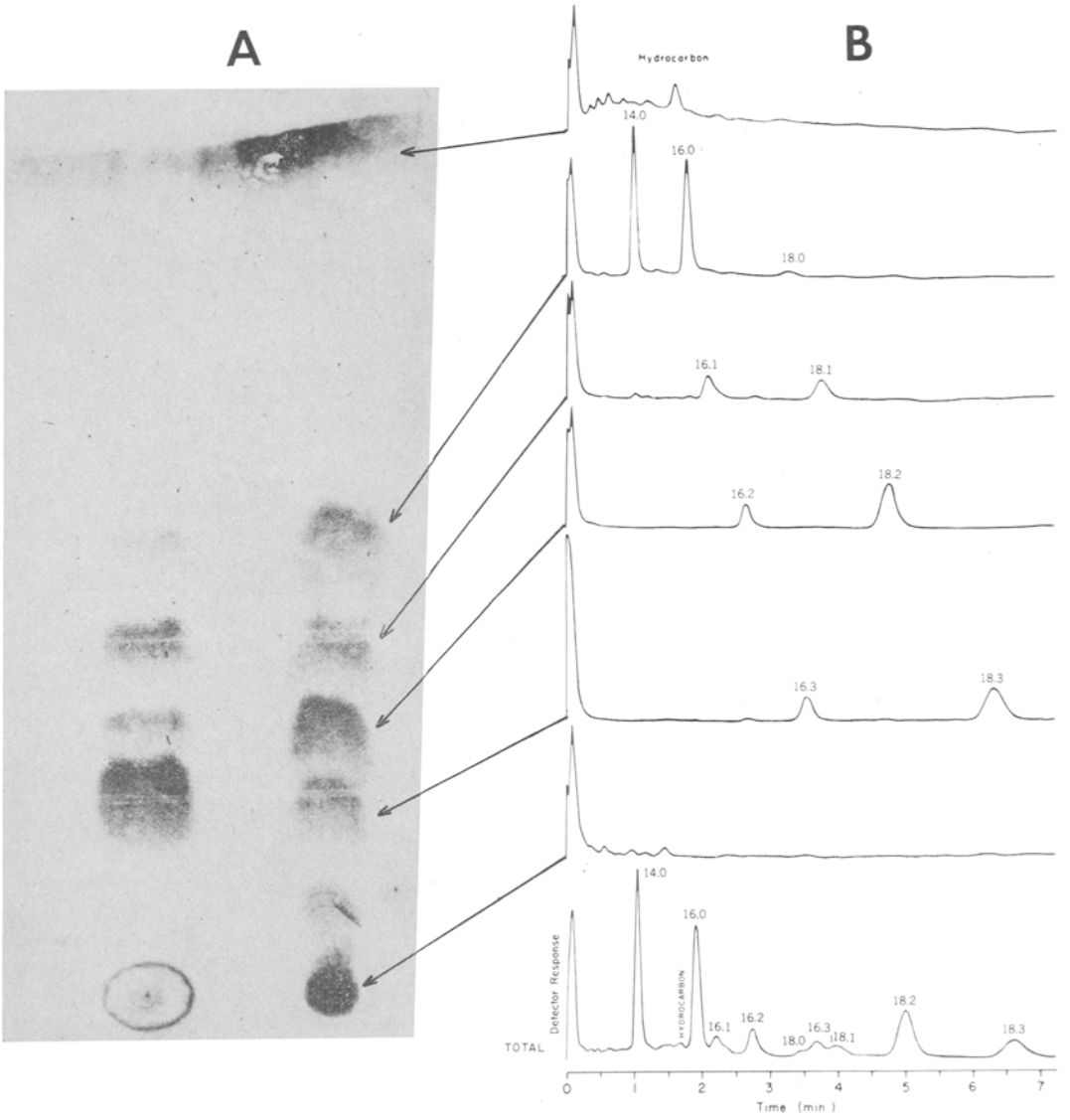

FIG. 1. The glass fiber paper chromatography (GFPC) and gas-liquid chromatography (GLC) identification of the fatty acid methyl esters of *C. pyrenoidosa:* A — A typical GFPC chromatogram, comparing the mobility of *Chlorella* fatty acid methyl esters (right) with that of a standard mixture (left). The standard mixture contains in order of decreasing mobility: methyl stearate, methyl oleate, methyl linoleate, methyl linolenate and arachidonate. B — Typical GLC chromatogram of algal fatty acid methyl esters eluted from a GFPC chromatogram. The bottom (total) is a GLC fatty acid pattern obtained from the total algal extract before separation of the fatty acids on GFPC. From left to right in the total GLC pattern appear methyl myristate (added as an internal standard), methyl palmitate, methyl palmitoleate, methyl hexadecadienoate, methyl stearate, methyl hexadecatrienoate, methyl oleate, methyl linoleate, and methyl linolenate.

chromatograph (Packard Instrument Company), equipped with a hydrogen flame detector and a 6-ft. × 4-mm (I.D.) coiled glass column, which was packed with 10% EGSS-X on 100/120 mesh Gas-Chrom P (Applied Science Laboratories). The column was operated at 165-175C with a N_2 pressure of 14 psi. The areas under the curves were measured by using a planimeter. Myristic acid, the internal standard, was used to calculate the con-

centrations of the *Chlorella* fatty acids quantitatively.

RESULTS

Extraction Efficiency

An experiment was carried out to check the efficiency of extraction of the fatty acids. One complete cycle of sonication, saponification, HCl neutralization, and hexane extraction (see Methods) were all considered as one "extraction." On the basis of sequential "extractions," 98% of the total fatty acids were accounted for in the first "extraction." These extractions were performed on "steady-state" cultures to ensure extractability of the fatty acids from cells at all stages of growth. An amount of cell material, well in excess of the usual experimental sample-size, was used in these experiments to ensure complete extraction of the samples obtained during the synchronous growth experiments.

Fatty Acid Identification

Fig. 1 illustrates the procedure used to identify the fatty acids. The fatty acid methyl esters were spotted in a continuous line across the bottom of a silica gel-coated glass fiber paper. The glass fiber paper chromatogram (ITLC-SG, Gelman Instrument Company) was developed with iso-octane, and the fatty acid bands were located by spraying with Rhodamine 6G. Each band was cut from the chromatogram and eluted with chloroform. Fig. 1A shows the separation of the algal fatty acids compared with a known mixture. It can be seen that the saturated monoenoic, dienoic, and trienoic methyl esters separate from one another; however it should be noted that the tetraenoic methyl ester, arachidonate, did not separate from the trienoic methyl ester, linolenate. The components in the chromatogram shown in Fig. 1A were detected by the H_2SO_4-char technique.

After the bands were eluted, they were subjected to GLC analysis. GLC tracings at the right of figure 1A are joined to the section of the glass fiber paper chromatogram from which they were eluted. In the total algal fatty acid pattern at the bottom it can be seen that the algal fatty acids are a mixture of C_{16} and C_{18} acids. In each series there are saturated monoenoic, dienoic, and trienoic fatty acids.

Schlenk et al. (19) have identified six of the eight major fatty acids in a "steady-state" culture (a culture containing cells of all ages) of *C. pyrenoidosa* (No. 7516 American Type Culture Collection). Table I compares the fatty acid data of Schlenk with that obtained from synchronous cultures of *C. pyrenoidosa* (7-11-05) at the zero and last hour of both experiments carried out in the present investigation. In the C_{16} fatty acid series, palmitic acid was the dominant fatty acid in both investigations. In the C_{18} series Schlenk et al. found oleate to be a dominant acid followed by linoleate, linolenate, and stearate whereas this investigation with *C. pyrenoidosa* (7-11-05) showed that linoleate was the dominant fatty acid followed by linolenate, oleate, and finally stearate.

It is interesting to note from the data in Table I that, regardless of small changes in percentage composition, the relative order of the composition in terms of concentration (i.e., 16:0 > 16:2 > 16:3 > 16:1/18:2 > 18:3 > 18:1 > 18:0) was almost unaffected by the growth conditions to which the cells were exposed throughout one complete growth cycle. Whether these patterns are altered during successive generations of growth under identical growth conditions or whether they are characteristic for *C. pyrenoidosa* 7-11-05 will be studied in future investigation.

TABLE I
A Comparison of the Fatty Acid
(Strain No. 7516 and No. 7-11-05) Composition of *C. pyrenoidosa*

		Percentage composition			
		Light nonglucose		Light glucose	
Fatty Acid	From Schlenk et al. (19)	0-hr	13-hr	0-hr	14-hr
16:0	13.6	30.7	41.0	34.3	47.7
16:1	3.2	5.5	4.1	7.9	4.2
16:2	7.0	9.1	7.5	8.1	6.1
16:3	5.1	6.8	6.3	7.2	4.6
18:0	3.5	3.0	1.6	2.4	1.7
18:1	34.7	7.4	5.2	7.0	6.8
18:2	17.7	24.9	23.0	21.9	21.2
18:3	14.6	13.1	11.1	11.2	7.9

Fig. 2 illustrates the synchronized cell division that was observed in "light-grown" cells in the presence and absence of glucose. In the absence of glucose the synchronized cell division was similar to that obtained in other laboratories with *C. pyrenoidosa* (6-8). In cultures grown with glucose, daughter-cell release was delayed by approximately two hours, and, in some cells, division was inhibited completely (as indicated by the fact that there was only approximately a three-fold increase in cell number). Microscopic examination of the culture during division revealed that there were many cells larger than the normal mother cells and that cytokinesis, when it occurred, resulted in the release of four daughter cells. Each "giant" mother cell was similar in appearance to those photomicrographed by Rodriguez-Lopez (20).

The log-linear increase in total cellular dry weight and phosphorus of cultures grown in the light in the absence of glucose (Fig. 3), has been established on numerous occasions (6,7,16). The dry weight (Fig. 3A) of cells exposed to glucose at two hours was equal to the dry weight of cells not exposed to glucose at three hours, indicating a faster rate of growth for the former. After four hours the rate of dry-weight increase in cells exposed to glucose dropped off and remained log-linear throughout the remainder of their growth cycle. The rate of increase was less than that observed for cells not exposed to glucose. These latter data were consistent with numerous reports (21) of the failure of exogenous energy sources to stimulate growth under conditions which are saturating for photosynthesis. The accelerated dry-weight increase during the initial four hours of growth was consistent with the hypothesis that glucose is directly utilized in starch biosynthesis (11). The rate of total cellular phosphorus increase of cells exposed to glucose (Fig. 3B), which probably would not reflect an enhanced starch biosynthesis, increased log-linearly through the eighth hour; thereafter the rate decreased (remaining log linear) throughout the period where cell division was inhibited.

A two-hour lag in the accumulation of the total fatty acids was observed and was followed by a nearly uniform increase through the sixth hour in photo-autotrophically grown cells (Fig. 4A). Between the sixth and seventh hours [about one hour before the onset of nuclear division (9)], the rate of increase of total fatty acids was appreciably depressed. Throughout nuclear division and cell division

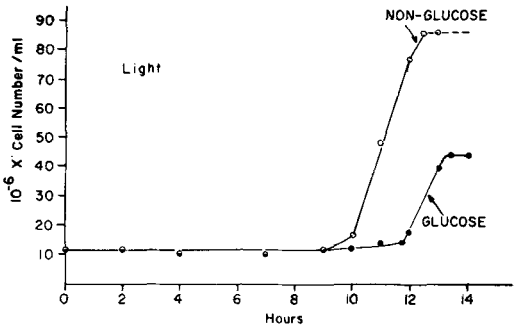

FIG. 2. A comparison of the cell concentration of *C. pyrenoidosa* during synchronous growth in the light in the presence and absence of glucose.

(seventh through 12.5 hour) the total fatty acid content of the cells increased uniformly. In the photoheterotrophically grown culture (Fig. 4B) there was a linear accumulation in total fatty acids until the fourth hour, at which time there was an abrupt increase until the sixth hour. Between the sixth and seventh hours of growth there was a marked decline in fatty acid accumulation, which was more pronounced than the decline observed in the absence of glucose during the same interval. An accelerated accumulation of total fatty acids was again observed during nuclear di-

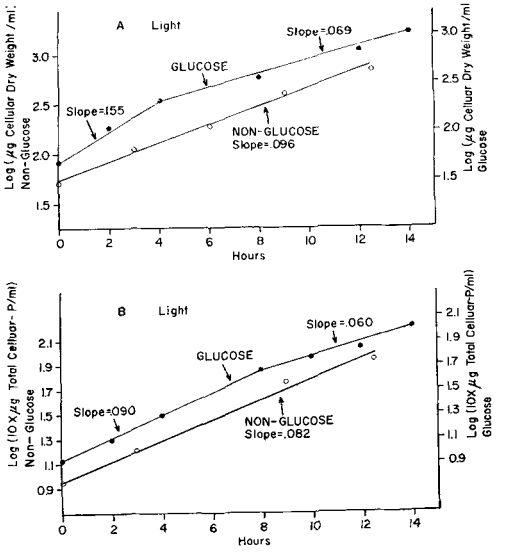

FIG. 3. A comparison of the increase in the total dry weight (A) and total cellular phosphorus (B) of synchronized cultures of *C. pyrenoidosa* grown in the light in the presence and absence of glucose.

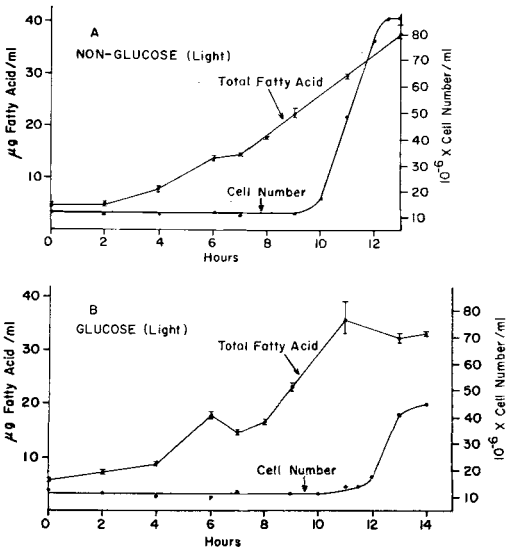

FIG. 4. Relationship between the total cellular fatty acids and cell concentration of synchronized cultures of *C. pyrenoidosa* grown in the light in the presence (B) or absence (A) of glucose.

vision, but, in contrast to the photo-autotrophically grown culture, the fatty acid accumulation ceased during cell division. The decrease in the rate of fatty acid accumulation during cell division of the cells exposed to glucose was not reflected in their dry-weight increase, which was log-linear from the fourth hour of growth (Fig. 3A). Thus, during growth of cells exposed to both light and glucose, the fatty acid concentration, rather than dry weight, seems more clearly to reflect the observed inhibition in cell division. Even though there were differences in accumulation curves, whether the cells were grown in the presence or absence of glucose, the total fatty acid content of the cultures after one growth cycle was nearly the same.

For the most part the changes in the individual fatty acids (Fig. 5) reflected the over-all trends in total fatty acid content (Fig. 4). There were several exceptions. In the photo-autotrophically grown cells only hexadecatrienoate and linolenate increased measurably during the first two hours of growth. After the first two hours palmitate increased at a log-linear rate, showing no decline during the sixth-seventh hour. Only palmitate and linoleate increased appreciably during cell division; the concentration of all other fatty acids remained essentially constant during the period. The concentration of each fatty acid, except palmitate, seemed to have a depressed rate of accumulation during the prenuclear division period. Linolenate showed the most marked depression.

The presence of glucose did not alter the similarities between the individual fatty acids (Fig. 5B) and the total fatty acid content (Fig. 4B). Most of the fatty acids increased during the first two hours of growth; the greatest increase was again observed in linolenate. In contrast to the photo-autotrophically grown cells nearly all fatty acids, including palmitate, declined markedly in concentration during the prenuclear division period. Each fatty acid increased during nuclear division and declined during cell division except for palmitate, which continued to increase during the latter stage of growth. Linoleate concentration increased considerably during cell division in photo-autotrophically grown cells; but in photoheterotrophically grown cultures the linoleate concentration dropped markedly during the cell division period.

DISCUSSION

Periodism in cytological (5) (such as nuclear and chloroplast division, cross-wall deposition, etc.) as well as metabolic (9,10) events (photosynthetic activity and endogenous respiration) during the maturation cycle of *Chlorella* would seem to require an accompanying periodism in biochemical components related to these events. This hypothesis has received support on numerous occasions (3,6,7,16,17) in synchronized cultures of *C. pyrenoidosa.*

The dramatic cytological changes in this organism, most of which occur in a two to three-hour period just before and during daughter-cell release, would seem to indicate that this period is one in which the energy-generating systems of the cell are operating at their maximum. Sorokin however has repeatedly demonstrated (8,10) that photosynthetic activity, which has been steadily declining since the fifth hour of growth, is approaching its lowest level during cell division. Since the major energy source of the organism operates minimally during this period, Duynstee (11) has postulated that, in order to meet its demands for energy- and/or carbon-building blocks, the cells must mobilize the carbohydrate and/or lipid reserves which have accumulated prior to this point. The decrease Duynstee observed (11) in the rate of total starch accumulation from the sixth hour of growth is consistent with its utilization as a

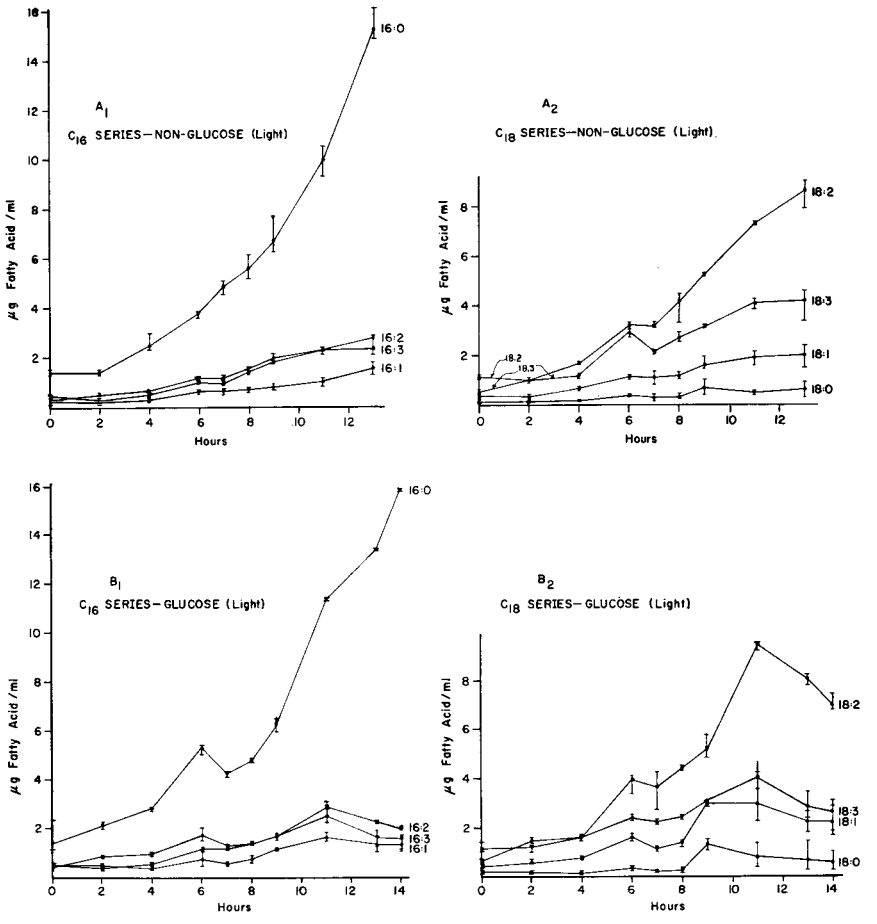

FIG. 5. A comparison of the individual fatty acid concentrations of synchronized cultures of *C. pyrenoidosa* grown in the light in the presence (B_1 B_2) and absence (A_1 A_2) of glucose.

carbon and/or energy reserve from the sixth hour of growth through cell division.

The role of the fatty acids as a possible contributor to the cell economy is not so clear. The rate of increase in the total cellular concentration of most of the fatty acids was depressed (sixth-seventh hour) just prior to nuclear (9) and chloroplast (5) division (seventh-tenth hour) and during cellular division (Fig. 5A). Linolenic acid was the only fatty acid to show an unequivocal net utilization during the prenuclear division period, suggesting perhaps more than a structural role for this lipid during this period. Palmitic acid, the dominant fatty acid in this organism, does not show a periodism during either the prenuclear or cell division periods but rather accumulates at an exponential rate (these data have been plotted but do not warrant an additional figure) from the second hour of growth throughout the life cycle. Since accumulation data are a net result of both synthesis and catabolism, it can only be concluded that the rate of palmitic acid synthesis always exceeds its rate of breakdown and that, since the accumulation is exponential, its rate of synthesis increases at least at the same exponential rate. By using ^{14}C labeling techniques it is hoped that future experiments will be able to assess the turn-over rate of these fatty acids.

In an attempt to elucidate the relative contributions of the fatty acids to an intracellular carbon energy pool, a second experiment was performed, in which glucose was supplied as an exogenous carbon and energy source to synchronously growing *Chlorella* cultures.

This experiment provided several interesting results. The lag in fatty acid accumulation

observed during the first two hours of growth in photo-autotrophically grown cultures was absent when the cells were grown in the presence of glucose (Fig. 4). Presumably this is a result of the fact that glucose supplies more direct precursors of fatty acid biosynthesis than do photosynthetic products. If such is the case, one would expect starch (17-20% of the cellular dry weight) rather than lipid content (3-4% of the cellular dry weight) more nearly to reflect a cellular demand for energy since the product of starch catabolism, glucose, should promote lipid biosynthesis rather than lipid catabolism. The net utilization of nearly every fatty acid during both the prenuclear and cell division stages of growth in cultures exposed to glucose (Fig. $5B_1 + B_2$) is however inconsistent with this suggestion. Rather than promoting fatty acid biosynthesis or at least lessening the rate of decline in fatty acid content, glucose administration promotes a net utilization of the fatty acids. The possibility that intracellular glucose plays a regulatory role in the utilization of intracellular fatty acids is an intriguing but speculative hypothesis from these data.

The observed inhibition of daughter-cell release observed in photoheterotrophically grown cultures, not unique to this laboratory (20), remains unexplained. The heterotrophic growth of *Chlorella* is currently under investigation in this laboratory.

The relationship of fatty acids, specifically α-linolenic acid and *trans*-3-hexadecenoic acid, to the Hill reaction in green plants and micro-organisms has been studied in several laboratories (2,12,13,14). The absence of these fatty acids from two species of blue-green algae which was nevertheless capable of carrying out the Hill reaction led Nichols (14) to conclude that the essential participation of either α-linolenic or *trans*-3-hexadecenoic acid in the Hill reaction can be discounted. Yet the lack of a universal distribution of these acids does not automatically preclude a unique association of these fatty acids with photosynthesis in the green algae or higher plants.

Sorokin (10) has reported that the photosynthetic activity in synchronized cultures of *C. pyrenoidosa* rises sharply during the first four hours of growth, peaks at this time, then progressively declines throughout the remainder of the life cycle. Though linolenate was the only fatty acid to show a measurable increase during the first two hours of photo-autotrophically grown cultures, its rate of accumulation is increased between the 4th and sixth hours of growth. After a brief prenuclear division drop in concentration, linolenate concentration then increases throughout the remainder of the life cycle. Thus linolenate, if related to the photosynthetic process at all, certainly has additional role(s) to play during cell development. It must be pointed out however that it is possible that only small amounts of the total linolenate in the cell are required for the photosynthetic function and that, once this requirement is met (i.e., during the initial period of growth), additional linolenate takes part in other cellular processes. The *trans*-3-hexadecenoic acid content of this organism, presumably represented by the shoulder on the 16:1 peak in the total algal fatty acids shown in Fig. 1A, represents such a small fraction of the total fatty acids in this organism (since it was always included in the estimate of 16:1) that its periodism during the growth cycle could not be reliably estimated from these data. It is unfortunate that the conditions necessary to rupture the organism will not permit subsequent isolation and chemical analysis of its organelles.

Results on the fatty acid pattern of *Chlorella* agree well with those of Schlenk (19) et al. with the exception of oleic acid. Aside from a possible strain difference between organisms, oleic acid content has been reported (22) to be drastically reduced in growth media similar to the authors', which have a high nitrogen content.

The role(s) which fatty acids play during cell maturation of this organism remain elusive. Observations (in triplicate) of the eight principal fatty acids under two different growth conditions do however seem to indicate their likely involvement in processes which occur during both the prenuclear and cell division stages of growth in *C. pyrenoidosa.*

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The Metabolism of C^{14}-α-Tocopheryl Quinone and C^{14}-α-Tocopheryl Hydroquinone

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ABSTRACT

The metabolism of C^{14}-d-α-tocopheryl quinone (α-TQ) and its hydroquinone (αTHQ) was investigated. Forty-six hours after intraperitoneal administration of either compound to rats the radioactivity isolated from the liver was present almost exclusively in C^{14}-α-TQ. The results indicated, however, that in situ this compound was present primarily in the reduced form. No conversion to C^{14}-α-tocopherol or other liver metabolites was observed. α-THQ was eliminated from the tissues more rapidly than α-TQ. The main metabolite excreted in the urine was a conjugate of α-tocopheronic acid and the main metabolite excreted in the feces was a conjugate of α-TQ. Free C^{14}-α-TQ was present in the feces after administration of this compound but not after C^{14}-α-THQ administration.

INTRODUCTION

ALTHOUGH THE FORMATION of *d*-α-tocopheryl-*p*-quinone (α-TQ) from *d*-α-tocopherol in animal tissues has been observed in several laboratories (1-3), little is known of its metabolic fate. The failure of large doses of this quinone to substitute for vitamin E in the rat fertility test (4-6) indicates that it is not cyclized in vivo to the chromanol form in this species. On the other hand, Mackenzie et al. (7) found that α-TQ had considerable biological activity toward the prevention and cure of muscular dystrophy in vitamin E deficient rabbits. Additional experiments by the Mackenzies (8, 9) on the biopotency of α-tocopheryl hydroquinone (α-THQ) demonstrated that this compound is active in the prevention of sterility and muscular dystrophy in rats when administered in daily doses, and they attributed previous negative findings to the use of too infrequent administration. From the fact that the large doses of α-THQ did not lead to the appearance of α-tocopherol in the serum of rabbits they also concluded that the hydroquinone is an antidystrophic factor per se (10). The conclusion drawn from other studies that the biological activity of vitamin E is attributable to its antioxidant properties (11, 12) implies that this is also true of α-THQ.

The present study was designed to obtain information on the metabolism of C^{14}-α-TQ and C^{14}-*d*-α-THQ in rats.

EXPERIMENTAL

Preparation of C^{14}-α-TQ and C^{14}-α-THQ

d-α-Tocopherol-5-CH_3-C^{14} (specific activity 1.3 μc/mg) was prepared from 10 mg of its succinate ester (Distillation Products Industries, Rochester, New York) by reductive cleavage with $LiAlH_4$ according to the procedure of Duggan (13). The purity of the product was established by paper chromatography and it was then dissolved in 20 ml of ethanol. Ten ml of 1% ethanolic dipyridyl and 10 ml of 1% $FeCl_3$ in ethanol were added and, after shaking for 20 min at 48C, the resulting hemiacetal was extracted with petroleum ether. After washing with aqueous $FeSO_4$ and water it was converted to the *p*-quinone by treatment with HCl in ethanol (14). The products were chromatographed on a 1.5 cm × 17 cm column of Bio-Rad neutral alumina (6% H_2O added) using increasing percentages of diethyl ether in petroleum ether (Skelly F, bp 30-60C) as developing solvents. A yellow band was eluted with 35% diethyl ether which contained 34 × 10^6 dpm (87% of the starting dpm). Upon paper chromatography of this material a single peak of radioactivity was observed which corresponded to standard α-TQ.

A portion of the pure C^{14}-α-TQ (2.7 mg) was emulsified by dissolving in 0.6 ml ethanol, adding 2.4 ml of isotonic saline containing 0.2% Tween 80, and shaking thoroughly. One ml (3 × 10^6 dpm) was administered intraperitoneally to each of 3 growing rats which had been fed a vitamin E deficient diets for 5 weeks. Another portion (3.6 mg) was dissolved in ethanol and reduced to C^{14}-α-THQ by adding solid KBH_4. The excess KBH_4 was removed by centrifugation and the remainder was emulsified in 4 ml of a similar mixture. One ml (3 × 10^6 dpm) was administered to

each of 3 deficient rats. The remaining C^{14}-α-TQ and C^{14}-α-THQ was used in the isolation procedure as an internal standard.

Experimental Animals

After injection, the experimental animals (body weight about 200 g) were placed in metabolism cages for 46 hr. The urine was collected under toluene and the feces in ethanol. These samples were stored at –15C pending analysis. The livers were removed under anesthesia, rapidly minced, quick-frozen by dipping in a dry ice-acetone bath and lyophilized. Pure C^{14}-α-TQ (15×10^4 dpm, equivalent to 5% of the dose) was mixed with the liver mince of two uninjected rats as an internal standard and a similar amount of C^{14}-α-THQ was added to the liver mince of two additional animals.

Liver Analysis

The dried tissue was ground to a powder and shaken twice with 50 ml of petroleum ether for 15 min. The residue from the combined extracts was dissolved in 15 ml of acetone and most of the lipid was removed by filtration after crystallization at –70C (15). The filtrate was evaporated to dryness under N_2 and the residue (19.4-46.6 mg) was dissolved in 3 ml of petroleum ether. Two to 3 mg of α-tocopherol, α-TQ, a tocopherol dimer and a trimer were added as carriers. The last three compounds have been identified in previous studies as metabolites of α-tocopherol in rat liver (16, 17). The mixture was applied to a 1.5 × 17 cm column of Bio-Rad neutral alumina (6% water added) which was developed with increasing proportions of ether in petroleum ether. Seventy fractions of 10 ml each were collected. Two ml of each fraction were used for the determination of C^{14} by liquid scintillation spectrometry. The other 8 ml were evaporated to dryness and the residue was dissolved in isooctane for the determination of optical density at the absorption maximum of the individual carriers.

Urine Analysis

The urine was extracted 3 times with 50 ml of ether and the combined extracts were washed once with 50 ml of saturated $NaHCO_3$ solution and twice with water. Aliquots of the ether phase were chromatographed on paper using 75% ethanol as mobile phase, silicone-coated paper using acetonitrile-water mixtures and on thin layers of Silica Gel G using 1% methanol in benzene and 3% methanol in cyclohexane. α-Tocopherol, α-TQ and α-tocopheronic acid were used as standards. A sample of tocopheronic acid, a urinary metabolite of α-tocopherol isolated by Simon et al. (18), was kindly provided by Dr. K. Schwarz.

As most of the radioactivity in the urine remained in the aqueous phase after ether extraction, acid and enzyme treatments were tested as possible means of hydrolyzing the water-soluble metabolites. Portions of the aqueous phase ($27\text{-}35 \times 10^4$ dpm) were made up to 3 N with concentrated HCl and refluxed for 2 hr. The ether extractable material then was chromatographed on an alumina column as described previously and the radioactive eluates were characterized by paper and thin layer chromatography. Additional portions of the aqueous phase, containing $8.8\text{-}14.0 \times 10^4$ dpm, were placed under vacuum to remove traces of ether and made up to 80 ml with H_2O. Ten ml of acetate buffer (pH 5.0) were added and the pH was adjusted to 4.6 with 9 N H_3PO_4 or solid $NaHCO_3$. Penicillin (250 units/ml) and streptomycin (25 μg/ml) were added and the mixture was divided into two parts. To one was added 20,000 Fishman units of bovine liver β-glucuronidase (Sigma Chemical Company); the other served as an enzyme blank. After incubating at 38C for 5 days the mixtures were extracted 3 times with ether and the labelled compounds were chromatographed against appropriate standards on thin layers of silica gel. Similar incubations were carried out using alkaline phosphatase (pH 10.5) and aryl sulfatase (pH 4.5).

Feces Analysis

The feces were filtered off, washed with ethanol, and extracted with petroleum ether in a Goldfish extractor for 12 hr. The petroleum ether and ethanol extracts were combined and evaporated to dryness in vacuo. The residue was extracted several times with petroleum ether and the insoluble portion was taken up in 10 ml of ethanol. The petroleum ether-soluble material was applied in 2 ml of solvent to a Bio-Rad alumina column along with 2-3 mg of α-tocopherol and α-TQ. The column was developed with ether-petroleum ether mixtures as described and the labelled eluates were chromatographed against known standards on thin layers of Silica Gel G. Thirty ml of H_2O was added to the ethanol-soluble fraction, the solution was made 3 N to HCl and the mixture was refluxed for 2 hr. The residue obtained by extracting 3 times

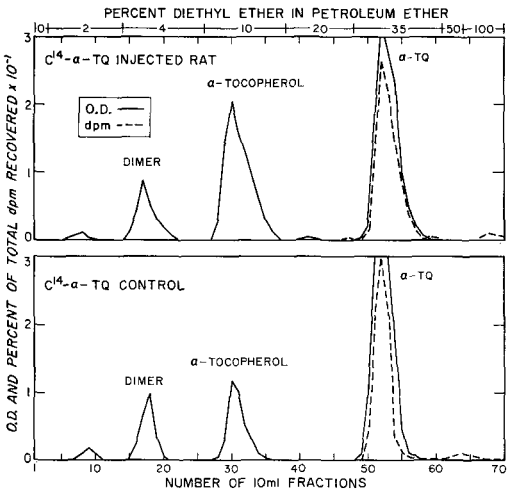

FIG. 1. Top: Elution profile for the radioactivity extracted from the liver of a C^{14}-α-TQ-injected rat after chromatography on a neutral alumina column.

Bottom: Elution profile for C^{14}-α-TQ internal standard. % dpm = dpm in fraction/total dpm recovered.

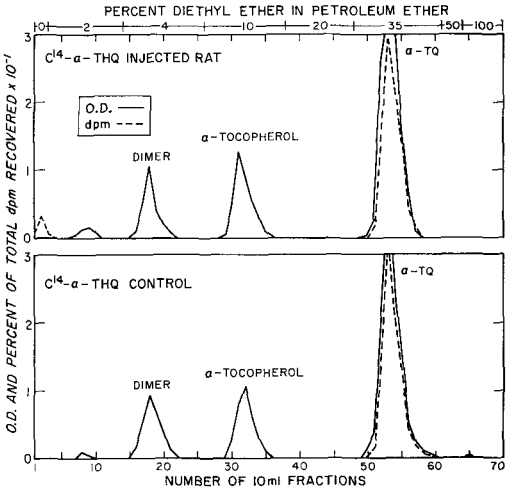

FIG. 2. Top: Elution profile for the radioactivity extracted from the liver of a C^{14}-α-THQ-injected rat after chromatography on a neutral alumina column.

Bottom: Elution profile for C^{14}-α-THQ internal standard. % dpm = dpm in fraction/total dpm recovered.

with petroleum ether and evaporating the solvent likewise was chromatographed on an alumina column and on Silica Gel G.

RESULTS

Liver Analysis

The results of chromatographing the liver extracts of the C^{14}-α-TQ-injected rats on alumina columns are illustrated in Figure 1. The only radioactive compound recovered in the eluates in significant amounts was C^{14}-α-TQ. The elution profile for the control shows that this compound was stable to the extraction and chromatographic operations used for its isolation. Similar results were obtained for the other injected and control animals. It is noteworthy that there was no conversion of the *p*-quinone to α-tocopherol or its dimeric metabolite.

A typical C^{14} elution pattern for the liver extracts of the α-THQ-injected rats is shown in Figure 2. The data show that, as in the case of the C^{14}-α-TQ-injected animals, the radioactivity recovered from the liver was almost entirely associated with the labelled quinone. The profile for the internal standard demonstrates, however, that the C^{14}-α-THQ was completely converted to the oxidized form in the course of the isolation procedure. Hence it is not possible to conclude from these data what relative amounts of labelled TQ and THQ were present in situ in the livers of the rats injected with either compound.

The identification of the C^{14}-labelled compound eluted with 35% ether as α-TQ was confirmed by thin-layer chromatography on silica gel G and by reduction with $SnCl_2$ to C^{14}-α-tocopherol. Single radioactivity peaks, determined by use of a Packard Radiochromatogram Scanner, were observed in the thin-layer systems at R_f values corresponding to those of standard α-TQ. The residue from the 35% ether fractions (Figure 1) was dissolved in 10 ml of 1,4-dioxane, 2 g of $SnCl_2$ and 1 ml of concentrated HCl were added and the mixture was refluxed for 1 hr. Then 15 ml of H_2O were added and the solution was extracted twice with 50 ml of ether. After washing the extract with water and evaporating the solvent, the residue was rechromatographed on an alumina column with α-tocopherol and α-TQ carriers. Development of the column as described previously yielded a major radioactivity peak which coincided with the α-tocopherol carrier, a lesser peak corresponding to carrier α-TQ and another small peak apparently representing a degradation product.

A summary of the radioactivity recovered

TABLE I

Recovery of Radioactivity from the Liver Extracts

Group	C^{14}-α-TQ-Injected			C^{14}-α-TQ Control		C^{14}-α-THQ-Injected			C^{14}-α-THQ Control	
Rat No.	1	2	3	4	5	6	7	8	9	10
DPM in liver extract										
Total (× 10^{-2})	512	1405	830	886	1022	61	64	92	898	975
% of dose	1.7	4.7	2.8	56[a]	68[a]	0.2	0.2	0.3	60[a]	64[a]
DPM after crystallization of lipids										
Total (× 10^{-2})	410	1226	767	819	957	41	49	78	831	895
% recovered	80	87	92	93	94	67	76	86	92	93
DPM in column eluates										
Total (× 10^{-2})	358	1150	723	793	852	31	35	58	683	717
% recovered	87	94	94	94	89	76	72	74	82	80
DPM in 35% ether fraction (C^{14}-α-TQ)										
Total (× 10^{-2})	308	1083	649	761	781	22	24	49	607	665
% of total eluted	86	94	90	96	92	72	68	84	89	93

[a] Percentage of dpm added to liver mince.

in the liver extracts is given in Table I. It is of interest that the amount of radioactivity present in the liver 46 hr. after injection of C^{14}-α-TQ was much greater (average of 3.1% of the dose) than after C^{14}-α-THQ injection (0.2%). This indicates a faster rate of metabolism of the hydroquinone and conforms with the observation that the biological response to this compound depends on its frequent administration (8, 9). The recovery of the internal standards in the extraction step was relatively low (average 63%), suggesting a binding of the labelled compounds to protein. Of the total dpm recovered in the column eluates, 90% were present in the TQ fractions from the rats injected with this compound and 75% from those injected with C^{14}-α-THQ.

Urine Analysis

The radioactivity recovered in the urine is summarized in Table II. The proportion of the dose excreted by this route ranged from 10.3 to 20.0% and was similar for the two groups of rats. Virtually all of the radioactivity (94-98%) in the urine was in an ether-insoluble form. After acid hydrolysis, 41-52% of the total dpm were extractable with ether, and when this radioactivity was chromatographed on alumina it yielded the elution profile shown in Figure 3. It is apparent that no α-tocopherol or α-TQ was released by hydrolysis and that most of the radioactivity (average 89.8%) was present in a polar compound(s) which was eluted in methanol-acetic acid. No difference was observed between the rats injected with C^{14}-α-TQ and C^{14}-α-THQ with respect to the distribution of radioactivity among the column eluates.

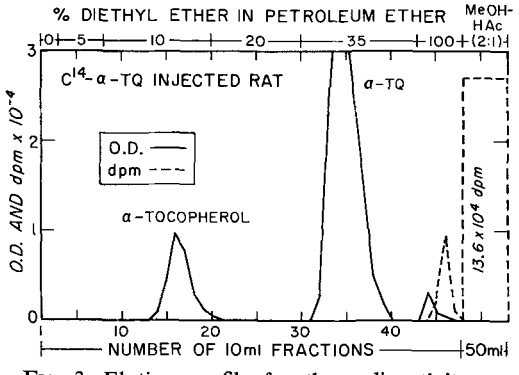

FIG. 3. Elution profile for the radioactivity extracted from the urine of a C^{14}-α-TQ-injected rat with ether after acid hydrolysis and chromatography on a neutral alumina column.

When the labelled urinary metabolites recovered by ether extraction before or after acid hydrolysis, or after digestion with β-glucuronidase, were chromatographed against pure standards on paper and thin-layer chromatograms, the results shown in Table III were obtained. The data confirmed the absence of α-tocopherol and α-TQ, and revealed that the radioactivity was largely, if not entirely, associated with a compound which migrated opposite α-tocopheronic acid. In the ether-soluble fraction of the unhydrolyzed urine, which contained 2-6% of the total dpm, and in the fraction obtained by enzyme hydrolysis, tocopheronic acid was the only C^{14}-compound present in significant amounts. In the acid hydrolysate additional peaks appeared in the scanogram which evidently represented acid degradation compounds.

Although significant amounts of free C^{14}-tocopheronic acid were released by the action of β-glucuronidase, as shown by comparison with the enzyme blanks, only a small fraction (5-7%) of the total ether-insoluble material

TABLE II

Radioactivity Excreted in the Urine of Rats During 46-hr Period after Injection with C^{14}-α-TQ or C^{14}-α THQ

	C^{14}-α-TQ-injected			C^{14}-α-THQ-injected		
Rat No.	1	2	3	4	5	6
Total dpm excreted ($\times 10^{-5}$)	3.90	4.27	3.09	3.28	3.19	5.98
% of dose	13.0	14.2	10.3	11.0	10.7	20.0
Dpm in ether extract ($\times 10^{-5}$)	.08	.09	.07	.15	.17	.24
Dpm in aqueous phase ($\times 10^{-5}$)	3.82	4.18	3.02	3.13	3.02	5.74

was hydrolyzed by this enzyme. Extension of the incubation time and alteration of the pH did not increase the yield of free acid. Phenolphthalein monoglucuronide added to the medium as an internal standard was completely hydrolyzed under the same conditions.

The possibility that the acid was present as an ethereal sulfate or phosphate ester was tested by treatment with aryl sulfatase or alkaline phosphatase as described. These enzymes yielded no labelled products which were extractable with ether. Hence, although most of the dpm in the urine were shown to be attributable to a conjugate of tocopheronic acid (of which at least a small portion was present as the glucuronide) the identity of the major conjugate was not established.

Feces Analysis

The total dpm recovered from the feces by ethanol and petroleum ether extraction amounted to 2.6-10.2% of the dose. The petroleum-ether-soluble fraction ranged from 31%-72% of the total radioactivity. No significant differences were found between the rats injected with C^{14}-α-TQ and C^{14}-α-THQ with respect to the total radioactivity excreted in the feces or the proportion of dpm in the petroleum ether fraction.

Representative elution profiles illustrating the results of chromatographing the residue from the petroleum ether fraction on alumina columns are shown in Figure 4. Most of the dpm extracted from the feces with petroleum ether (59-77% and 79-92%, respectively, from the rats injected with C^{14}-TQ and C^{14}-α-THQ) were recovered in the methanol-acetic acid fraction. A clear peak of radioactivity (15.3-31.1% of the total dpm eluted) was associated with the α-TQ carrier in the case of the rats which were injected with this compound, whereas only 1.6-3.5% of the radioactivity was eluted in this fraction in the case of the C^{14}-α-THQ-injected group. The identity of the labelled compound was confirmed by thin-layer chromatography against pure α-TQ on silica gel G using 1% methanol in benzene (R_f = 0.55) and 3% methanol in cyclohexane (R_f = 0.45) as solvents. Reduction with $SnCl_2$ and rechromatography on a neutral alumina column led to recovery of 37.4% of the radioactivity in the α-tocopherol fraction. These results established the presence of a small amount of free C^{14}-α-TQ (5.2-7.3% of the total dpm) in the feces of the C^{14}-α-TQ-injected rats and showed that the amount of this compound in the feces of the C^{14}-α-THQ-injected animals was negligible.

Hydrolysis of the petroleum-ether-insoluble fraction of the feces extract with 3 N HCl for 2 hr converted 45-52% of the dpm into a soluble form. Chromatography of this portion on a neutral alumina column yielded the results shown in Figure 5. Most of the dpm

TABLE III

R_f Values for C^{14}-Compounds Obtained from Urine by Ether Extraction, Acid Hydrolysis or Treatment with β-glycuronidase

		Standards			*Urinary compounds obtained by*		
Solid phase	Liquid phase	α-Toc.	α-TQ	α-Toc. acid[a]	Ether extraction	Acid hydrolysis	β-glucuronidase treatment
Paraffin-coated paper	75% ethanol	0.2	0.5	0.9-1.0	0.9	0.9-1.0	....
Silicone-coated paper	20% acetonitrile	0	0	0.7-0.8	0.7-0.8	0.7-0.8	....
Silicone-coated paper	50% acetonitrile	0	0	1.0	1.0	1.0	....
Silicone-coated paper	70% acetonitrile	0.3	0.4	1.0	1.0	1.0	....
Silicone-coated paper	90% acetonitrile	0.8	0.8	1.0	1.0	1.0	....
Silica gel thin layer	1% MeOH in Benzene	0.8	0.5-0.6	0.4-0.6	0.4-0.5	0.4-0.6	0.5
Silica gel thin layer	3% MeOH in Cyclohexane	0.5	0.4-0.6	0.4-0.6	0.5-0.6	0.5-0.6	0.4

[a] α-tocopheronic acid.

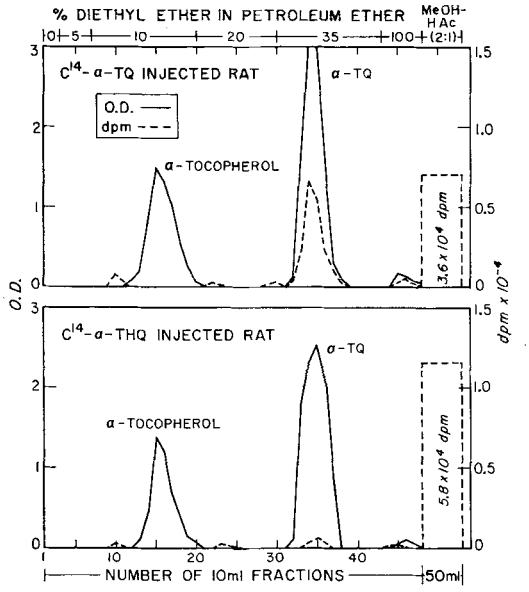

FIG. 4. Elution profile for the radioactivity in the petroleum-ether-insoluble fraction of the feces of a rat injected with C^{14}-α-TQ (top) and C^{14}-α-THQ (bottom) after chromatography on a neutral alumina column.

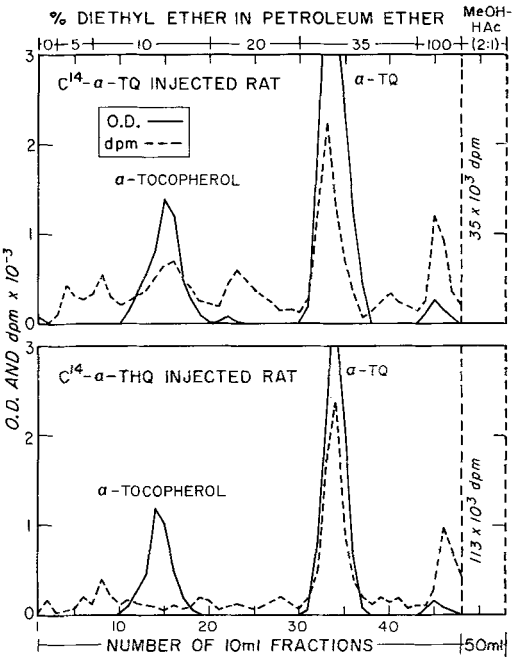

FIG. 5. Elution profile for the radioactivity in the petroleum-ether-insoluble fraction of the feces of a rat injected with C^{14}-α-TQ (top) and C^{14}-α-THQ (bottom) after acid hydrolysis and chromatography on a neutral alumina column.

were recovered in methanol-acetic acid; however, a small peak is discernible in the 35% ether fractions which was associated with the quinone carrier. The presence of the labelled quinone was confirmed by thin layer chromatography and reduction to C^{14}-α-tocopherol. Of the total dpm recovered in the column eluates, 12.5% and 13.3% of those derived from the feces of two C^{14}-α-TQ-injected rats was attributable to α-TQ. The corresponding values for two animals injected with C^{14}-α-THQ were 4.6% and 5.5%.

The identity of the petroleum ether-soluble fecal compound(s) which was eluted from alumina with methanol-acetic acid (Figure 4) is unknown; possibly it is synonymous with the conjugate present in greater amounts in the petroleum-ether-insoluble fraction. β-glucuronidase failed to hydrolyze it. A portion of the radioactivity eluted in the non-TQ fraction after acid hydrolysis of the petroleum-ether-insoluble fraction (Figure 5) is attributable to artifacts. Treatment of TQ with HCl under the conditions used for hydrolysis, followed by chromatography on alumina, revealed the presence of degradation products which were eluted in these fractions. Other products may have been formed as a result of the action of intestinal microorganisms.

DISCUSSION

Although it was not possible to determine directly whether the oxidized or reduced form of α-TQ was the predominant species present in the liver in situ, the occurrence of conjugates in the feces and urine implies that this compound was present mainly in the reduced state in the liver and kidneys. A large fraction of the radioactivity in the feces of the C^{14}-α-THQ-injected rats was present as a conjugate which apparently was formed from the reduced compound in the liver. No consistent evidence was obtained for the presence of the oxidized form in the feces of these animals. By contrast, the feces of the C^{14}-α-TQ-injected rats contained significant amounts of the free quinone. This material probably represented that portion of the dose which was secreted in the bile before it could be reduced and conjugated in the liver.

The reduction of quinones by liver enzymes is well known. In addition to ubiquinone, a natural consituent of the electron transport system of mitochondria, synthetic compounds such as menadione and benzoquinone have been shown to be actively reduced in vitro by nicotinamide adenine dinucleotide-catalyzed

reductases (19). Reduction of α-TQ by a pyridinoprotein enzyme occurs in spinach chloroplasts (20). If α-TQ is readily reduced in mammalian tissues, however, some explanation is needed for its low biological activity relative to that of the hydroquinone. It is possible that the progressive enzymatic reduction of α-TQ is closely coupled with conjugation of the reduced compound. A single parenteral dose of α-THQ, on the other hand, may exert an antioxidant effect in the peripheral tissues before it is deactivated by conjugation in the liver and degradation in the kidneys.

The finding that the urine contained a major metabolite of α-TQ in the form of an α-tocopheronic acid conjugate supports the scheme proposed by Simon and coworkers (18) for formation of this acid from α-tocopherol. It also accounts for the observation that after C^{14}-α-tocopherol administration to rats only traces of radioactivity (< 1% of the dose) were found in the urine (1). Since there is only a limited conversion of α-tocopherol to α-TQ in vivo, and of the amount of α-TQ formed only part is excreted by way of the urine, it is evident that α-tocopheronic acid is, under normal conditions, a minor metabolite of vitamin E. Schmandke (21) has reported that 0.3% of a dose of 1 g of α-TQ administered orally to a human subject was excreted in the urine as a conjugate of α-tocopheronic acid. This low conversion is probably a reflection of inefficient absorption from the intestine.

The resistance of the urinary tocopheronic acid conjugate(s) to hydrolysis with β-glucuronidase is surprising in view of the contrary observation of Simon et al. (18). Although hydrolysis with this enzyme clearly liberated a small amount of free acid, the metabolite(s) remained largely in a bound form and was released only by acid hydrolysis.

The results of this and previous studies suggest a general scheme for the metabolism of α-TQ in animal tissues. This compound is apparently a product (along with a dimer and trimer) of the oxidation of α-tocopherol by lipid free radicals or peroxides. It is reduced to α-THQ in the liver, conjugated with glucuronic acid and other compounds, and is secreted in the bile. It appears in the feces largely in a conjugated form along with a small amount of free quinone which escaped conjugation in the liver. In the kidney α-TQ undergoes reduction, conjugation and oxidative degradation of the sidechain to form a conjugate of α-tocopheronic which is excreted in the urine.

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Digestion of Butyrate Glycerides by Pancreatic Lipase[1,2]

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ABSTRACT

The racemic triglycerides, glyceryl-1-palmitate-2,3-dibutyrate (PBB), glyceryl-1-butyrate-2,3-dipalmitate (PPB), glyceryl-2-butyrate-1,3-dipalmitate (PBP), and the diglyceride, racemic glyceryl-1-palmitate-3-butyrate (P-B) were synthesized and digested with pancreatic lipase. Each triglyceride was mixed with equimolar amounts of triolein (OOO) prior to incubation.

The following order of digestion rates was observed: PBB > PPB > PBP > P-B. There was no evidence for short-chain fatty acid specificity; however the triglycerides containing butyric acid were hydrolyzed more rapidly than OOO. Based upon the fatty acid composition of partial glycerides, digestion of butyrate glycerides was not a simple phenomenon. For example, in the digestion of PBB, butyric acid accumulated faster than palmitic acid in the diglycerides, and monobutyrin was found to accumulate when the diglyceride, P-B, was digested. As evidenced by the fatty acid composition of the monoglycerides, positional specificity of pancreatic lipase was always maintained.

INTRODUCTION

MILK LIPASE was found to digest glycerides in a manner almost identical with pancreatic lipase (1). Nevertheless one major difference appeared to exist between these otherwise similar lipolytic enzymes. Entressangles and co-workers (2) concluded that pancreatic lipase possessed a short-chain fatty acid specificity, but this laboratory found that milk lipase did not differentiate between a short- and long-chain fatty acid attached to the primary positions of the same triglyceride (3).

This difference was confusing since it had been observed that both of these lipases yielded similar fatty acid patterns in the products of lipolysis from the digestion of milk fat, namely, large quantities of butyric acid (4:0) in the free fatty acids and lesser quantities of 4:0 in the residual triglyceride as compared with the original triglyceride. Thus, contrary to expectations, the milk enzyme gave results which could be interpreted as short-chain fatty acid specificity.

At that time it was not understood how milk lipase could preferentially release butyric acid from the mixed natural fat and not from a synthetic triglyceride, such as racemic glyceryl-1-palmitate-2,3-dibutyrate (PBB). These paradoxical results were reconciled when the milk enzyme was incubated with an equimolar mixture of the synthetic triglycerides, PBB and triolein (OOO), and greater quantities of both palmitic acid (16:0) and 4:0 compared with oleic acid (18:1) were released but not more 4:0 relative to 16:0 (4). In considering lipolysis of the butyrate triglyceride, there was not a preferential digestion of the 4:0 linkage but instead a more rapid hydrolysis of the entire triglyceride as compared with OOO. This differential rate of lipolysis between glycerides was termed intermolecular specificity, as contrasted to fatty acid specificity or intramolecular specificity, but is more correctly defined as nonrandom hydrolysis of glyceride classes.

The results of the lipolysis of milk fat by the milk enzyme could now be attributed to intermolecular specificity for the butyrate triglycerides, not necessarily to a simple preferential digestion of the butyrate ester bonds. It was a necessary, logical step to ask whether the data reported for the pancreatic lipolysis of short-chain fatty acid triglycerides were in actuality the result of intermolecular specificity and not short-chain fatty acid specificity. Indeed, the authors are convinced that this is the case and have already presented some preliminary data which support this hypothesis (5). Nevertheless data in the literature (2) can only be interpreted as short-chain fatty acid specificity, suggesting that these results with PBB may have been a special case. Therefore the research reported herein was initiated to explore more fully the behavior of pancreatic lipase in the hydrolysis of butyrate glycerides.

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MATERIALS AND METHODS

Substrates

Triolein (OOO) was purchased from the Hormel Institute (Austin, Minn.) and purified by elution through alumina (6). Racemic glyceryl-1-palmitate-2,3-dibutyrate (PBB), racemic glyceryl-1-palmitate-3-butyrate (P-B), and glyceryl-2-butyrate-1,3-dipalmitate (PBP) were prepared by reacting 1-monopalmitin with the appropriate quantities of acid chloride. The synthesis of PBP involved the intermediate, 1,3-dipalmitin. All intermediates and final products, including the 1-monopalmitin, were synthesized according to established procedures (7,8).

Racemic glyceryl-1-butyrate-2,3-dipalmitate (PPB) was prepared by reacting the diglyceride, P-B, with palmitoyl cholride or by reacting glyceryl-1,2-dipalmitin with butyryl chloride (7); the dipalmitin was synthesized from palmitoyl chloride and 1-tetrahydropyranyl glyceryl ether essentially as described by Krabisch and Borgstrom (9) and was purified by crystallization from petroleum ether (30-45C) at room temperature.

PBB, PBP, and PPB were purified by elution through alumina (6). P-B was purified by procedures outlined by Mattson and Volpenhein for 1,3-diglycerides (7) except that the temperature of crystallization was –25C. As determined by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) (8,10), the purity of all intermediates and final glycerides, including the OOO, was estimated to approach 99%.

Digestion Conditions

Each of the butyrate triglycerides (1.05 millimoles) was mixed with an equimolar quantity of OOO, 56 ml of 0.25 molar Tris buffer (pH 8.0) containing 10% w/v gum arabic, 3.5 ml of 4 molar $CaCl_2$, and 1.4 ml of 1% w/v bile salts. The mixtures were emulsified with the aid of a Waring Blendor and distributed so that each digestion flask contained approximately 300 micromoles of substrate, 8 ml of Tris buffer, 0.5 ml of $CaCl_2$, and 0.2 ml of bile salts. Samples were incubated in the presence of 25 mg of pancreatic lipase for 2.5, 5, and 10 min at 37C. The diglyceride substrate, P-B, was treated in essentially the same manner except that no OOO was used; 2.5- and 15-minute incubation periods were employed, and each sample contained 600 micromoles of substrate and 50 mg of pancreatic lipase. Duplicate samples of each substrate was analyzed at each time-interval. In addition, one control containing no enzyme was incubated for each substrate at the longest time-interval employed. The enzyme preparation and the general enzymatic procedure have been described (11).

Extraction and Analysis

Samples were extracted with chloroform-methanol (9:1) by a procedure adapted from Harper et al (12). Digestion mixtures were acidified with 0.5 ml of 20% H_2SO_4 and carefully rinsed with water and ethyl ether into a casserole containing activated silicic acid. The sample was triturated and mixed with 75 ml of extracting solvent; the resulting slurry was transferred to a column fitted with a sintered glass disc. The sample was eluted from the silicic acid with the aid of a vacuum from a water aspirator. Before the column was dry, the casserole was rinsed with 175 ml of solvent, and this was used to elute the sample completely. After extraction the samples were divided into two equal portions. One portion was separated by preparative TLC (5) into residual triglyceride, diglyceride, monoglyceride, and free fatty acids; the other portion was used to determine 4:0 in the free fatty acids as described below. Fractions separated by TLC were converted to butyl esters and analyzed by temperature-programmed GLC (10). Free fatty acids were esterified by refluxing in butanol with H_2SO_4 as the catalyst (13); neutral lipids were esterified with sodium butoxide in the presence of dibutyl carbonate (10).

Estimation of Butyric Acid in the Free Fatty Acids

One-half of the extracted sample was first titrated to the thymol blue end-point to determine total free fatty acids. Then the solvent was removed with the aid of a rotary flash evaporator, ca 10 mg of 4:0 were added to facilitate discrimination during chromatography, and the contents were acidified and separated by conventional column chromatography according to the AOAC procedure for the estimation of 4:0 (14). The long-chain fraction was collected and titrated; 4:0 was calculated by the difference between the total titration and the long-chain titration. The ratio of 16:0 to 18:1 in the latter fraction was obtained from GLC data. Several "digestion mixtures" containing known amounts of 4:0 and 16:0 were prepared and conducted through the extraction and free fatty acid estimation steps to determine the recovery of 4:0 by this method. Prior to extraction, each of these samples was mixed with 0.5 ml of $CaCl_2$, 0.2

TABLE I

Recovery of Standard Mixtures[a] of Butyric and Palmitic Acid

Samples analyzed	Fatty Acid Added 16:0	Fatty Acid Added 4:0	Fatty Acid Recovered[b] Total[c]	16:0[d]	4:0[e]	Mole Percentage 4:0 Added	Found	Recovery
	Microequivalents			Percentage				
8[f]	74.6	77.7	99.3	93.4	105.3	51.0	54.1	106
3	287.9	58.0	90.9	89.8	95.5	16.8	17.6	105
3	375.4	48.9	97.3	95.0	115.1	11.5	13.6	118
2	198.4	73.9	90.0	91.3	86.6	27.1	26.1	96
2	151.0	198.0	91.6	99.3	86.0	56.7	53.2	94
2	149.8	152.2	100.1	98.9	101.2	50.4	51.0	101
1	223.0	325.0	97.9	101.2	95.1	59.3	57.8	98
1	263.3	146.6	92.7	91.3	95.4	35.8	36.8	103
Average % recovery			95.0	95.0	97.5	....	....	103

[a] These were mixed with 0.5 ml of $CaCl_2$, 0.2 ml of 1% bile salts, and 10 ml of 10% gum arabic in pH 8.0 Tris buffer.

[b] These are averages of number of samples analyzed.

[c] Recovery after extraction step is shown.

[d] Recovery includes extraction and column chromatography.

[e] The 4:0 contents were calculated by difference [total free fatty acids-long-chain fatty acids].

[f] These samples also contained 125 mg of purified olive oil triglycerides.

ml of 1% bile salts, and 10 ml of pH 8.4 Tris buffer containing 10% gum arabic, then was allowed to incubate with shaking at 37C for 5 min. Eight of these samples also contained 125 mg of purified olive oil triglycerides (6). A total of 22 samples was analyzed. Data concerning the ratios and microequivalents of fatty acid added as well as the recovery values are recorded in Table I.

RESULTS AND DISCUSSION

As can be seen in Table I, the recoveries for 4:0, on an absolute basis, varied from 86-115% but, on the average, were slightly higher than those for 16:0. This can be attributed to the extra step of column chromatography required for the determination of 16:0. Butyric acid, in this method, can be lost only before the total titration step, thus the recovery of 4:0 relative to 16:0 is generally high (last column, Table I) and averages 103%. The addition of olive oil triglycerides did not appreciably alter the recoveries.

The recovery of total fatty acids in this silica gel extraction method was 95% and was comparable with those previously reported (12). No attempt was made during this study to ascertain the recoveries of each individual digestion product; however, based on previous work (15,16), the average recoveries of all products were judged to be 90% or greater. An estimate of the relative recovery of the triglycerides can be made by comparing the compositions of these fats from the intact and enzymeless controls (Table II). The values for the intact fat were from GLC analyses of butyl esters of an aliquot of the digestion mixture prior to extraction whereas those for the enzymeless control were based on similar analyses after extraction and TLC. Some loss of oleate triglyceride did occur (average recovery = 97.7%) and may have been caused by oxidation during TLC as noted by Nichaman et al. (17). From data in Tables I and II it was concluded that the method was suitable for the analyses under investigation.

The data from the pancreatic lipase digestions are summarized in Tables III-VI as mole percentage of each product in each fraction from TLC. The results from the triglyceride mixtures are compared with theoretical values calculated from control samples by assuming the absence of both fatty acid and intermolecular specificity. In the runs involving PBB and OOO (Table III) it was apparent that PBB had been preferentially hydrolyzed as, after 10 minutes of digestion, only 16.9 M% remained compared to an initial value of 52.9 M%.

TABLE II

Fatty Acid Composition of Synthetic Triglycerides Before[a] and After[b] Extraction and Thin-Layer Chromatography

Sample		4:0	16:0	18:1
		Mole percentage		
PBB + OOO[c]	Before	33.1	16.9	50.0
	After	35.3	17.5	47.2
PPB + OOO[c]	Before	16.2	32.4	51.4
	After	16.5	32.9	50.6
PBP + OOO[c]	Before	17.4	34.7	47.9
	After	16.6	35.2	48.2

[a] Before = intact TG sampled immediately after mixing triolein with the butyrate triglyceride.

[b] After = enzymeless control sample.

[c] P = palmitate, B = butyrate, O = oleate.

TABLE III

Some Lipolytic Products from Equimolar Mixture of PBB + OOO[a]

Minutes	Residual Triglyceride		Diglyceride Mole percentage		
	PBB	OOO	PB–	–BB	–OO
2.5	45.2	54.8	20.8	52.1	27.1
5.0	29.2	70.8	21.6	48.7	29.7
10.0	16.9	83.1	13.6	46.6	39.8
Theory	52.8	47.2	26.4	26.4	47.2
Minutes	Monoglyceride		Free fatty acid		
	–B–	–O–	P	B	O
2.5	50.0	50.0	35.9	31.3	32.8
5.0	61.6	38.4	33.5	28.1	38.4
10.0	58.4	41.6	28.2	28.8	43.0
Theory	52.8	47.2	26.4	26.4	47.2

[a]These were incubated with 25 mg of pancreatic lipase at 37C; P = palmitate, B = butyrate, O = oleate.

TABLE V

Some Lipolytic Products from Equimolar Mixture of PBP + OOO[a]

Minutes	Residual Triglyceride		Diglyceride Mole percentage	
	PBP	OOO	PB–	–OO
2.5	49.8	50.2	53.6	46.4
5.0	49.8	50.2	47.1	52.9
10.0	44.5	55.5	49.6	50.4
Theory	51.8	48.2	51.8	48.2
Minutes	Monoglyceride		Free fatty acid	
	–B–	–O–	P	O
2.5	73.7	26.3	61.9	38.1
5.0	58.7	41.3	58.9	41.1
10.0	56.2	43.8	55.7	44.3
Theory	51.8	48.2	51.8	48.2

[a]These were incubated with 25 mg of pancreatic lipase at 37C; P = palmitate, B = butyrate, O = oleate.

This decrease in butyrate triglyceride could have resulted from specificity for either triglyceride or fatty acid. Examination of the free fatty acid data however eliminates the possibility of a simple specificity for butyric acid which would require a greater than theoretical content of 4:0. Instead, both 16:0 and 4:0 were released in greater than theoretical amounts. These results are inconsistent with a specificity for butyric acid but can be explained by a more rapid hydrolysis of the butyrate triglyceride with equal lipolysis rates of either primary ester linkage.

The pattern in the partial glycerides is more difficult to interpret. The accumulation of monobutyrin was expected since the butyrate triglyceride was being preferentially digested, and monobutyrin is hydrolyzed relatively slowly compared with longer-chain monoglycerides (18). The diglyceride values however were unexpected. The high values of dibutyrin may be attributable to the partial solubility of this diglyceride in water, which resulted in a preferential digestion of the PB diglyceride. This is partially substantiated by the higher amounts of 16:0 relative to 4:0 in the 2.5- and 5.0-minute free fatty acids.

In Table IV are summarized the results for the digestion mixtures of PPB-OOO. Again, butyrate triglyceride has been preferentially digested, and the free fatty acid data do not support a simple concept of short-chain fatty acid specificity. As for PBB, the diglyceride values are difficult to interpret. The BP diglyceride relative to the dipalmitin should be present in equal amounts; however, at all times sampled, there was relatively more BP present. Also in the free fatty acid data, relatively more 16:0 than 4:0 was released. These results are consistent with a faster digestion rate for the PP diglyceride. As for PBB, it was the diglyceride containing the least butyric acid which appeared to be digested most rapidly and is contrary to what would have been predicted. Similar results would be expected if the natural substrate for pancreatic lipase was the 1,2-diglyceride as compared with the 1,3-diglyceride and if preferential acyl migration occurred so that the diglycerides which contained more butyrate isomerized to the 1,3-diglycerides the fastest. However numerous alternative hypotheses could be formulated, all of which would be equally speculative.

TABLE IV

Some Lipolytic Products from Equimolar Mixture of PPB + OOO[a]

Minutes	Residual Triglyceride		Diglyceride Mole percentage		
	PPB	OOO	BP–	–PP	–OO
2.5	48.7	51.3	37.9	24.5	37.6
5.0	45.1	54.9	44.8	16.1	39.1
10.0	35.2	64.8	34.5	20.1	45.4
Theory	49.4	50.6	24.7	24.7	50.6
Minutes	Monoglyceride		Free fatty acid		
	–P–	–O–	B	P	O
2.5	47.6	52.4	28.5	31.6	39.9
5.0	50.0	50.0	23.4	36.8	39.8
10.0	48.5	51.5	23.6	28.2	48.2
Theory	49.4	50.6	24.7	24.7	50.6

[a]These were incubated with 25 mg of pancreatic lipase at 37C; P = palmitate, B = butyrate, O = oleate.

TABLE VI

Some Lipolytic Products from Glyceryl-1-Palmitate-3-Butyrate[a]

Minutes	Monoglycerides[b]		Free fatty acids[b]	
	Mole percentage			
	—B—	—P—	B	P
2.5	58.0	42.0	39.3	60.7
15.0	66.1	33.9	39.1	60.9

[a]The 240 mg per sample were incubated at 37C with 50 mg of pancreatic lipase.

[b]B=butyrate, P=palmitate.

The results from the PBP-OOO mixtures are displayed in Table V. Evidence for a preferential attack of the butyrate triglyceride was most pronounced in the 10-minute residual triglyceride, the monoglyceride, and the free fatty acids. In the digestions of PBP-OOO it was possible to detect no 4:0 in the free fatty acids and only traces (less than 1 M%) of monopalmitin. These results with PBP contrast those of Clement et al. (19), who found 25 M% 4:0 in the free fatty acids when this glyceride was digested with human pancreatic lipase. However the latter digestions were conducted for two hours as compared with the 10-minute digestions in this study. Undoubtedly acyl migration is a serious problem in lengthy digestions since Clement et al. reported only 2 M% 4:0 in the free fatty acids when the digestions were limited to 20 minutes.

The absence of 4:0 in the free fatty acids and the virtual absence of 16:0 in the monoglyceride derived from PBP in this study are good evidence that the positional specificity of pancreatic lipase can be maintained during digestions involving butyrate glycerides. Also the absence of any 4:0 in the free fatty acids excludes the possibility that the preferential hydrolysis of this butyrate triglyceride is attributable to a short-chain fatty acid specificity.

Some of the results from the digestion of the diglyceride, P-B, are presented in Table VI. In the free fatty acid more 16:0 rather than 4:0 is present, a finding which is in contrast to what has been reported (2). From these results it would be predicted that the pattern in the monoglyceride should be the opposite of the free fatty acid values, that is, if more 16:0 was liberated, then more 4:0 should remain behind in the monoglyceride. This was indeed observed as monobutyrin accumulated.

These results would be expected if the diglyceride isomerized during the digestion process and if this isomerization involved the preferential migration of the 4:0 from the primary to the secondary hydroxyl. The 4:0 present as the secondary position ester would be less available to the pancreatic lipase. However, when these digestion mixtures were checked by boric acid TLC (20) for the presence of 1- and 2-monoglycerides, only the 1-isomer was observed. Furthermore, in experience with synthetic glycerides, the 1,3-diglyceride has been found to be much more stable than the 1,2-diglyceride.

A more plausible explanation for the results in Table VI assumes the preferential digestion of 1-monopalmitin. This is understandable when it is recognized that monobutyrin is water-soluble and that pancreatic lipase is known to act at a water-oil interface (21). Therefore monobutyrin might be relatively unavailable to this enzyme. This is supported by the report (18) that monobutyrin was hydrolyzed more slowly than monolaurin.

In the digestion of P-B it should be pointed out that, compared with the triglyceride substrates, the diglyceride was not hydrolyzed readily. A comparison of the 2.5-minute values, based on the ueq of fatty acid released per minute per 25 mg of enzyme gave: PBB-OOO, 63.4; PPB-OOO, 52.7; PBP-OOO, 40.0; and P-B, 17.5. Similar differences have been noted when other triglycerides and diglycerides were compared (18,22). Nevertheless, when digestion rates of diglycerides were determined in a triglyceride $\rightarrow$ diglyceride $\rightarrow$ monoglyceride reaction (16,23), the diglycerides and triglycerides appeared to be digested at comparable rates. This may simply reflect differences in ease of emulsification, but it is relevant that the natural substrate for pancreatic lipase is the 1,2-diglyceride formed at the interface by lipolysis. Therefore comparisons drawn from separate incubations of 1,2- or 1,3-diglycerides may not be valid.

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Human Placental Lipid Metabolism. III. Synthesis and Hydrolysis of Phospholipids

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ABSTRACT

Both diacyl GPC (glycerylphosphorylcholine) and diacyl GPE (glycerylphosphorylethanolamine) are synthesized in human placental tissue from their respective monoacyl precursors. The origin of the monacyl phosphatides is apparently not the result of placental phosphatide acyl-hydrolase activity. The most likely source is maternal serum. The declining level of 1-acyl GPC in maternal serum is not attributable to lysophosphatide acyl-hydrolase activity and is probably explained by placental utilization for the synthesis of diacyl GPC.

INTRODUCTION

THE ACTIVITY in placental tissue of acyl GPC acyl-transferase (E.C. 2.3.1.99) (1) has been previously described. This study reports the synthesis of diacyl GPE from 1-acyl GPE. In placental tissue the monoacyl substrate could arise from phosphatide acyl-hydrolase (E.C. 3.1.1.4) activity. Therefore the hydrolysis of endogenous and exogenous diacyl phosphatides was measured. Another explanation is that acyl GPC may be derived from maternal serum, as was originally suggested by Svanborg and Vikrot (2). The decline in acyl GPC during pregnancy (2) could represent either utilization or hydrolysis by the placenta. The placental activity of lysophosphatide acylhydrolase (E.C. 3.1.1.5) was therefore measured.

MATERIALS AND METHODS

Placental tissue was perfused and prepared as previously described (1). These preparations are referred to as the tissue homogenate.

The 1-acyl GPC and 1-acyl GPE were prepared from chicken egg yolk (3). The phosphorus to ester value of 1-acyl GPC was 0.97 and of 1-acyl GPE, 0.92. *Beta* 1-^{14}C-diacyl GPC and *beta* 1-^{14}C-diacyl GPE were prepared with ^{14}C-oleic acid by the enzymatic action of human erythrocytes on 1-acyl GPC and 1-acyl GPE (4).

The conversion of 1-acyl GPE to diacyl GPE was measured by incubating the homogenate with 1-acyl GPE, CoA (coenzyme A), ATP (adenosine triphosphate), and 1-^{14}C-oleic acid.

Phosphatide acyl-hydrolase activity was measured by incubating the homogenate with *beta* 1-^{14}C-diacyl GPC or *beta* 1-^{14}C-diacyl GPE. The hydrolysis of endogenous diacyl phosphatides was measured by determining the decline of diacyl GPC and diacyl GPE phosphorus values. Lysophosphatide acyl-hydrolase activity was determined by measuring the disappearance of added 1-acyl GPC phosphorus. The level of endogenous acyl GPC was too low in this system to measure any hydrolysis.

The lipids from all incubations were extracted and the lipid fractions separated by thin-layer chromatography on silica gel or column chromatography on silicic acid. All substrates used were purified by silicic acid column chromatography and appeared pure on thin-layer chromatography. The ^{14}C was measured by a Nuclear Chicago gas flow-counter to a 5% accuracy level.

In all instances "phosphate buffer" refers to a 0.1 N, pH=7.4 buffer containing one micromole per milliliter of Na deoxycholate. When erythrocytes were used the cells were washed and the hemolysate was used undiluted.

RESULTS

Previous studies showed the synthesis of diacyl GPC from 1-acyl GPC (1). Incubations were performed by using 1-acyl GPE as the substrate. When erythrocytes, ^{14}C-oleic acid, and 1-acyl GPE are incubated, 44% of the radioactivity is incorporated into diacyl GPE (Table I) with no incorporation in the diacyl GPC fraction. However when perfused placenta is incubated with ^{14}C-oleic acid and 1-acyl GPE, 18% of the radioactivity is incorporated into diacyl GPE and 23% into diacyl GPC. In the absence of 1-acyl GPC or 1-acyl GPE no radioactive diacyl phosphatides were formed by placental tissue. The synthesis of diacyl GPC when 1-acyl GPE was the substrate could be explained by the conversion of diacyl GPE to diacyl GPC. However the incubation of ^{14}C-diacyl GPE with placenta

TABLE I
Synthesis of Diacyl GPE and Diacyl GPC from 1-acyl-GPE[a]

Time (minutes)	Percentage of C^{14} Incorporated			
	Erythrocyte hemolysate		Perfused placenta	
	Diacyl-GPE	Diacyl-GPC	Diacyl-GPE	Diacyl-GPC
0	2	2	2	2
5	4	1	7	6
10	7	1	13	13
15	10	2	17	20
20	13	2	18	19
60	44	4	18	23

[a] Incubation mixture consists of 1-^{14}C-oleic acid (26 millimicromoles, 116,000 cpm), 400 millimicromoles of 1-acyl-GPE, 0.1 ml of 0.1 M $MgCl_2$, 0.4 ml of phosphate buffer, 0.1 ml ATP (10 micromoles), 0.2 ml of tissue homogenate, and 0.2 ml CoA (26 millimicromoles). Figures represent the percentage of ^{14}C in each lipid fraction.

yielded no ^{14}C-diacyl GPC. Also incubation of perfused placenta with S-Adenosyl-1-Methionine-Methyl-^{14}C in the system of Gibson et al. (5) gave no ^{14}C-diacyl GPC whereas a crude homogenate of rat liver did lead to ^{14}C-diacyl GPC. The possibility must be considered that the substrate, 1-acyl GPE, may be contaminated with 1-acyl GPC and that placental acyl-GPC acyl-transferase differs from the erythrocyte enzyme in its affinity for 1-acyl GPE. There was no evidence however from the thin-layer chromatography that there was contamination of the 1-acyl GPE substrate.

The hydrolysis of endogenous diacyl GPC and diacyl GPE was measured, and the results are shown in Table II. In three hours there is no appreciable hydrolysis of either endogenous substrate. When 14C-diacyl phosphatides are incubated with placental tissue, there is slight hydrolysis of diacyl GPE in three hours and no hydrolysis of diacyl GPC (Table III). The control incubation with rat intestine homogenate shows almost complete hydrolysis.

The incubation of 1-acyl GPC with perfused placenta showed no significant hydrolysis (Table IV) whereas hydrolysis in this system

TABLE II
Absence of Hydrolysis of Endogenous Diacyl GPC and Diacyl GPE[a]

Time (minutes)	Endogenous Phospholipid	
	Diacyl GPC	Diacyl GPE
0	45.1	30.5
60	43.7	29.2
120	41.1	29.2
180	42.4	29.2

[a] Incubation consists of 1.4 ml of placental homogenate and 0.6 ml of phosphate buffer. Figures represent the average of triplicate incubations expressed as millimicromoles of phosphorus in total incubation.

TABLE III
Hydrolysis of Diacyl Phosphatides[a]

Tissue	Percentage of ^{14}C in Lipid Fractions		
	Fatty acid	Diacyl phosphatide	Acyl phosphatide
Incubation with ^{14}C-Diacyl GPC			
No enzyme	2	95	3
Rat intestine	86	12	2
Perfused placenta	4	92	4
Incubation with ^{14}C-Diacyl GPE			
No enzyme	9	89	2
Rat intestine	93	5	2
Perfused placenta	17	82	1

[a] Incubation mixture consists of 0.2 ml of ^{14}C-diacyl GPC labeled in the 2 position (160 mum, 10,000 cpm) or ^{14}C-diacyl GPE labeled in the 2 position (99 mum, 9,400 cpm) sonicated in phosphate buffer, and 0.2 ml of the tissue homogenate. All incubations were stopped at three hours. Figures represent the average percentage of ^{14}C found in the lipid fractions from duplicate incubations.

does occur with rat intestine homogenate.

In these experiments where little or no hydrolysis occurred, the placental homogenate showed adequate phosphatide synthesis when cofactors and substrate were added (as in Table I) and was not inactivated by the method of preparation.

DISCUSSION

This work has shown that placental tissue can convert 1-acyl GPC (1) and 1-acyl GPE to diacyl GPC and diacyl GPE respectively. The question arises whether or not the monoacyl substrate is produced in the placental tissue by phosphatide acyl-hydrolase activity. Since in these experiments there is no hydrolysis of endogenous diacyl GPC and endogenous diacyl GPE, no hydrolysis of exogenous diacyl GPC and only slight hydrolysis of exogenous diacyl GPE, it seems unlikely that placental phospholipase activity affords the 1-acyl substrate for synthesis of the diacyl

TABLE IV
Absence of Hydrolysis of 1-Acyl GPC by Placenta[a]

Time (minutes)	Tissue		
	No enzyme	Placenta	Rat intestine
0	2.75	2.48	2.39
10	2.61	2.67	1.95
20	2.39	2.53	1.49
40	2.70	2.72	0.96

[a] Incubation mixture consists of 0.2 ml of tissue homogenate, 0.4 ml of 1-acyl GPC solution (4 micromoles), and 0.4 ml of phosphate buffer. At each designated time 0.2 ml were removed and analyzed. Figures represent micromoles of phosphorus in 1-acyl GPC fraction of total incubation and are the average of triplicate incubations.

phosphatides. These results are different from those of Popjak (6), who injected ^{32}P-labeled phospholipids into rabbits and recovered ^{32}P-glycerophosphate and inorganic ^{32}P from the placenta. He concluded that this represents placental breakdown of phospholipids. However no tissue incubations were reported.

The monoacyl substrate may arise from the maternal blood, and a decline in maternal serum acyl GPC does occur during pregnancy (2). This decline cannot be explained by placental hydrolysis of acyl GPC since these studies show that this activity is absent in perfused placenta. The absence of lysophosphatide acyl-hydrolase activity differs from the results of Winkler, who showed this activity in rat placenta preparations (7). The variation between the results may be explained by the action of erythrocytes, which are known to have lysophosphatide acyl-hydrolase activity (8), or by species or incubation differences. The maternal origin of the monoacyl substrates is further suggested by the work of Eisenberg et al., who have shown that the rat placenta removes 1-acyl GPC from the maternal circulation and utilizes it to form placental phospholipids (9).

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Comparison of Column Chromatographic Methods for the Quantitative Determination of Mono- and Digalactodiglycerides in Fresh Alfalfa *(Medicago sativa)*

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ABSTRACT

Three column chromatographic procedures for separating and recovering the galactolipids in fresh alfalfa extracts were compared. Silicic acid chromatography yielded pure fractions by thin-layer chromatography, infrared absorption, and chemical analysis. The carbon-Celite column gave the highest yield of monogalactodiglyceride. Of the 1.2% total lipids of fresh alfalfa, approximately 12% was monogalactodiglyceride and 8% was digalactodiglyceride. Linolenic acid accounted for about 90% of the total fatty acids in these components.

INTRODUCTION

O'BRIEN AND BENSON (1) used three columns in series (Florisil, DEAE-cellulose, silicic acid) to separate the galactolipids in fresh alfalfa extracts. This system facilitated the separation of the sulfolipid, but the amounts of monogalactodiglyceride (MGDG), 4.6%, and digalactodiglyceride (DGDG), 8.6% recovered, differed from those obtained by the authors with dehydrated alfalfa lipids (MGDG, 11.7%; and DGDG, 6.5%) by silicic acid column separation (2). Reported here is a reinvestigation of these two methods with a lipid extract from fresh alfalfa, also of results obtained with still a third column chromatographic technique.

MATERIAL AND METHODS

Fresh alfalfa (*Medicago sativa*) was cut in the field (U.C. Experimental Station, Albany, Calif.), immediately frozen by contact with dry ice, and kept frozen with dry ice until extracted on the same day by the method of Zill and Harmon (3) (successive extractions with boiling 95% ethanol, cold acetone, and chloroform-methanol, 2:1). The combined extracts were evaporated to dryness in vacuo and dissolved in ether. The ether solution was washed with water, the water wash was back-extracted with ether, and the ether extracts were combined. Yield of total lipid was 1.2% of fresh weight. Aliquots of the same alfalfa extract were used for each of three different column chromatography methods.

Method A. This included separation of the major fractions on a preparative silicic acid column, followed by rechromatography of each major fraction on a second silicic acid column. Silicic acid which had been obtained prewashed, according to the method of Hirsch and Ahrens (4) (Bio-Rad, Richmond, Calif.), was further washed with methanol to remove residual ether, then with chloroform to remove the methanol. It was dried and activated at 100C for 12-16 hr. A slurry of 300 g in 3:1 C-M (chloroform-methanol by volume; the chloroform contained 0.5% methanol added after distillation) was poured into 5 x 50-cm column, packed under 2.5 psi N_2 pressure, and washed with chloroform to remove methanol. The alfalfa extract (1 g lipid in 10 ml 9:1 C-M) was then applied, and the fractions were eluted with about 10 liters of 9:1 C-M, then with one to two liters each of 1:1 C-M, and with methanol.

The galactolipid fractions, as determined by thin-layer chromatography (TLC) and infrared (IR) spectra, were rechromatographed on 50 g of silicic acid in a 2.5 x 50-cm column, prepared as described above. The stepwise elution gradient used was 500 ml each of 9:1 C-M, 3:1 C-M, 1:1 C-M, and methanol. MGDG was eluted with 9:1 C-M; DGDG, with 3:1 C-M.

Method B. This was described by O'Brien and Benson (1), who used Florisil, DEAE-cellulose, and silicic acid columns in succession.

Method C. The absorbent was a carbon-Celite preparation. One hundred grams of 07-352 Nuchar-Attaclay (Varian Aerograph) were slurried in chloroform and poured into a 4.5 x 50-cm column. The height was 18 cm. One gram of lipid extract in chloroform was applied to the column, which was eluted with one liter each of chloroform 9:1 C-M, 3:1 C-M, 1:1 C-M, and methanol. The 9:1 C-M eluted MGDG; and 1:1 C-M, DGDG. Eluates were free from chlorophylls or other green-brown pigments but contained some

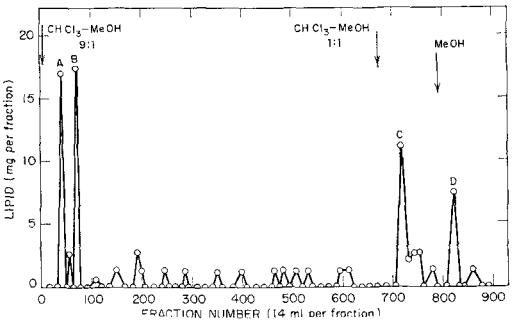

Fig. 1. Elution pattern of alfalfa lipids from silicic acid. (See text for conditions and peak identification.)

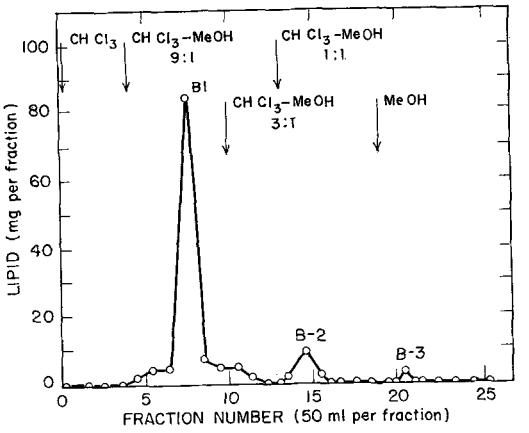

Fig. 2. Elution pattern obtained by rechromatography of Peak B (cf. Fig. 1).

carotenoids. MGDG and DGDG fractions were rechromatographed on silicic acid columns, as described above.

Galactolipid fractions were analyzed for galactose by the method of Bailey (5). Phosphorus was determined by the method of Bartlett (6). Fatty acid composition was determined by gas chromatography of the methyl esters which were obtained by transesterification of the fractions with methanol that contained 1% sulfuric acid. An F&M Model 810, equipped with dual columns with hydrogen detectors, was used. Two 6-ft columns, 5 mm ID, packed with 10% DEGS (diethylene-glycol-succinate, Varian Aerograph) on Chromsorb Q 80-100 mesh (Applied Science Laboratories, College Station, Pa.), were employed. The column temperature was 186C, and the N_2 flow was 75 ml/min. The columns were equilibrated at 200C for two days and standardized with NIH standards or equivalent (Applied Science Laboratories), as described by Horning et al. (7).

The TLC method of Nichols (8) was used to determine qualitatively the amounts of impurities in each fraction; quantitative results were obtained, according to the method of Blank et al. (9), with a Photovolt densitometer (Photovolt Corporation, New York, N. Y.).

TABLE I

Silicic Acid Chromatography of Alfalfa Lipids. Method A

Eluting Solvent	Peak No.	Yield mg	Recovery[a] %
Original (cf. Fig. 1) 1.004 g			
9:1 C-M	A	196.4	19.5
9:1 C-M	B	138.5	13.8
1:1 C-M	C	98.1	9.8
MeOH	D	57.4	5.7
.....	Others	425.4	42.4
Total		915.8	91.2
Peak B (cf. Fig. 2), 138.5 mg			
9:1 C-M	B-1	117.5	84.8
1:1 C-M	B-2	15.0	10.8
MeOH	B-3	2.5	1.8
Total		135.0	97.4
Peak C (cf. Fig. 3), 98 mg			
3:1 C-M	C-1	65	66.0
1:1 C-M	C-2	20	20.5
MeOH	C-3	10	10.5
Total		95	97.0

[a] As percentage of starting material.

RESULTS

Method A (Silicic Acid Chromatography)

The fractionation of crude alfalfa lipids is shown in Fig. 1. Peaks A, B, C, and D accounted for about 50% of the starting material, about 40% was eluted as minor peaks, and 9% was not eluted (Table I). The usual lipid loss on silicic acid columns has been found to be not more than 2 to 3% of the starting material. By TLC analysis the major

TABLE II

R_f Values by TLC of Mono- and Digalactodiglyceride (MGDG and DGDG) from Peaks B-1 and C1

Solvent System[a]	B-1 (MGDG)	C-1 (DGDG)
a	0.63	0.20
b	0.32	0.07
c	0.83	0.37
d	0.97	0.82

[a] a=C-M-H_2O (90:12:1.5).
b=C-M (95:12).
c=C-M-acetic acid-H_2O (90:12:1.5).
d=C-M-NH_4OH-H_2O (65:37.5:8:2.75).

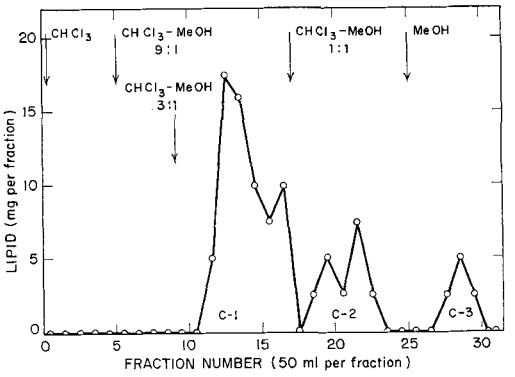

Fig. 3. Elution pattern obtained by rechromatography of Peak C (cf. Fig. 1).

components of peak A were simple lipids and of peak D were phospholipids; no fractions were homogeneous. Peaks A and D were not studied further.

Peak B was rechromatographed on a second silicic acid column (Fig. 2). About 97% of the starting material was recovered, 85% in the first peak (B-1, Table I). Peak B-1 was MGDG by TLC (Table II) and infrared absorption (Fig. 4) analysis. Fraction B-2 was mainly DGDG.

Peak C (Table I) was also rechromatographed (Fig. 3). Total recovery was 97%; 66% was in peak C-1 (Table I). This fraction was DGDG by TLC characteristics (Table IV) and infrared absorption (Fig. 5).

The MGDG (B-1, Table I) and the DGDG (B-2, and C-1, Table I) fractions contained no detectable phosphorus and 19.2% (theor. 23.1%) and 33.3% (theor. 38.2%) galactose respectively. The low values reflect galactose destruction during hydrolysis. The principal fatty acid was linolenic acid (Table III), which is characteristic for galactolipids in higher plants (Table VII). These preparations of

TABLE III

Fatty Acid Composition[a] of alfalfa Galactolipids Prepared by Three Column Chromatographic Methods

	Method A		Method B		Method C	
Fatty acid	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG
C16:0	3.5	7.0	1.8	6.2	1.8	6.1
C16:1	1.0	2.3	0.1	tr	tr	0.2
C17:0	....	....	tr	....	0.4	0.3
C18:0	0.7	2.6	0.3	2.5	0.3	0.4
C18:1	0.9	2.3	0.2	0.2	0.4	0.3
C18:2	2.7	0.7	1.8	1.2	1.9	0.9
C18:3	91.2	83.8	95.7	89.9	95.0	91.8

[a] Each peak expressed as percentage of the total peak area.

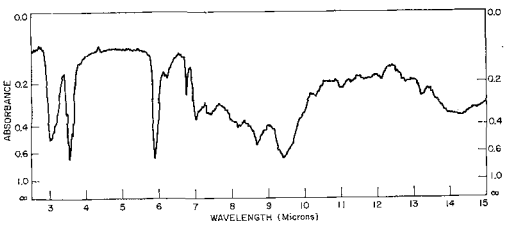

Fig. 4. Infrared absorption spectra of monogalactodiglyceride.

MGDG and DGDG were used for comparison with fractions obtained by the two other column methods.

Method B (Florisil, DEAE Cellulose, and Silicic Acid Chromatography) (1)

The galactolipid fraction obtained after Florisil and DEAE-cellulose chromatography accounted for 17% of the total lipid. It was further fractionated on silicic acid to separate the MGDG and DGDG from pigments and simple lipids. Table IV shows the results obtained. The simple lipids were not completely separable. The amount of MGDG in the simple liquid fraction (Fraction 1, Table IV) was found by quantitative TLC densitometry to be 43% (12 mg). The simple lipids of Fraction 1 were mainly triglycerides and pigments. Fraction 6 contained polar material, the TLC properties of which were similar to those of oxidized lipids. The two main fractions from the silicic acid column were MGDG (part of Fraction 1 and most of Fractions 2 and 3) and DGDG (Fractions 4 and 5). Fatty acid analysis (Table III) showed the expected high content of linolenic acid, and the IR and TLC characteristics were similar to those of the samples mentioned above.

TABLE IV

Silicic Acid Rechromatography of the Galactolipid Fraction from DEAE-Cellulose Column. Method B

Fraction	Elution solvent	Weight mg	Yield[a] %	Identity[b]
1	9:1 C-M	28.0	17.1	Simple lipids + MGDG[c]
2	9:1 C-M	30.0	18.3	MGDG
3	9:1 C-M	19.3	11.8	MGDG
4	4:1 C-M	34.2	20.9	DGDG
5	4:1 C-M	29.6	18.1	DGDG
6	MeOH	3.1	1.9	Oxidized lipids (?)
Total		144.2	88.1	

[a] Percentage of starting material; weight of starting material was 163.4 mg.

[b] Identity determined by TLC and IR comparison of characterized fractions (see text).

[c] 43% MGDG by quantitative TLC (densitometry).

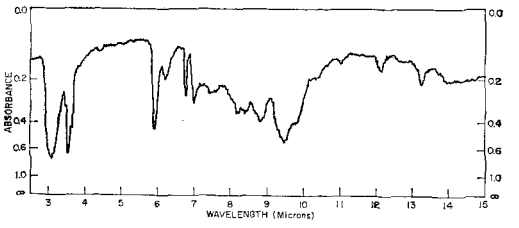

Fig. 5. Infrared absorption spectra of digalactodiglyceride.

The amount of material recovered from the silicic acid column (88%) was less than that obtained by rechromatography of the galactolipid fractions (97%, Table I). Possibly the acid wash procedure which was used in this case might have altered the column characteristics of the silicic acid enough to cause increased hydrolysis or possibly oxidation on the column. Other than in Fraction 1, the MGDG and DGDG preparations were essentially homogeneous. Only traces of impurities could be seen with overloaded TLC plates.

Method C (Nuchar-Attaclay Chromatography)

In an attempt to develop a simpler column system, a packing of carbon-Celite (Nuchar-Attaclay), extensively used to remove plant pigments prior to insecticide analysis (10), was investigated. It was hoped that galactolipids would be eluted from the column and the pigments retained. Results are shown in Table V.

Fraction 1 gave a typical TLC pattern for simple lipids and contained some yellow pigments; it was not further characterized. The MGDG and DGDG fractions also had small amounts of yellow pigments and other impurities, but these were readily removed by rechromatography on silicic acid columns. MGDG was obtained in 70% yield (140 mg) from Fraction 2, Table V; DGDG in 79% yield (41 mg) from Fraction 3, Table V. Both MGDG and DGDG fractions were homogeneous by TLC and gave typical IR and fatty acid analyses (Table III). The linolenic acid content was higher in this DGDG preparation than in those obtained by the other methods.

Fractions were free from chlorophyll products, but the low total yield (44.5%) makes the Nuchar-Attaclay column impractical for quantitative recovery of lipid constituents other than MGDG and possibly DGDG. The bulk of the material (60%) eluted from the column was in the galactolipid fractions. It is possible that modifications of this procedure may make possible a single chromatographic column for the quantitative separation of MGDG and DGDG.

TABLE V
Elution from Nuchar-Attaclay Column. Method C

Fraction	Elution solvent	Amount eluted (mg)	Yield[a] %	Identity
1	$CHCl_3$	154	16.4	Simple lipids
2	9:1 C-M	200	21.3	MGDG (yellow)
3	3:1 C-M	52	5.5	DGDG (yellow)
4	MeOH	12	1.3	Uncharacterized
Total		418	44.5	

[a] As percentage of starting material, 941 mg.

TABLE VI
Yields of Galactolipids from Fresh Alfalfa Extract[a]

Method	MGDG %	DGDG %	Total %
A. Silicic acid	11.7	8.0	19.7
B. Florisil, DEAE-cellulose, silicic acid	6.5	6.7	13.2
Data of O'Brien and Benson (1) by Florisil, DEAE-cellulose, silicic acid	4.6	8.6	13.2
C. Nuchar-Attaclay	14.8	4.4	19.2

[a] Of the total extractable lipids.

DISCUSSION

Since the same lipid extract was used for all the methods which were investigated in the present study, the yields of MGDG and DGDG are directly comparable (Table VI).

Method B of O'Brien and Benson (1) gave poorer yields of MGDG than did the other two methods. Further drawbacks were in the incomplete separations on silicic acid rechromatography and the large elution volumes required to elute the MGDG fraction. The yield of DGDG reported in this paper is similar to those reported by O'Brien and Benson.

The highest yields of MGDG were obtained by Method C, but this method gave the poorest yield of DGDG. DGDG was not eluted completely by the solvent system used.

TABLE VII
Linolenic Acid Content[a] of MGDG and DGDG in Various Plant Lipids

	MGDG %	DGDG %
Alfalfa leaves (present study, average)	94	89
Alfalfa leaves (O'Brien and Benson) (1)	95	82
Red clover (Weenink) (12)	75	83
Runner-bean (Sastry and Kates) (13)	96	93
Spinach (Allen et al.) (11)	97	84

[a] As per cent of total fatty acids.

Method A (silicic acid) gave higher values of DGDG but did not separate the galactolipids cleanly on the preparative column. In preliminary studies not reported in this paper, the use of a less polar-eluting solvent initially did not result in better separation. A continuous instead of a stepwise gradient might have yielded improved results.

Recently Allen et al. (11) described a method which uses counter-current separation prior to DEAE cellulose chromatography. Increased yields, little pigment contamination, and clean separations were reported.

Of the two galactolipids, MGDG was the more difficult to isolate, by the methods employed, without losses. Preliminary observations indicate that MGDG may be more labile to autoxidation than DGDG; the losses hence may be attributable to oxidation.

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Polarography of Conjugated Unsaturated Lipids[1]

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ABSTRACT

Conjugated fat-soluble vitamins, methylenic interrupted and conjugated fatty acids were polarographically investigated in both basic and neutral solvents. The half-wave potentials of all-*trans*-retinol, 13-*cis*-retinol, all-*trans*-retinyl acetate, all-*trans*-retinal, and Vitamin D_2 and D_3 were related to the number of double bonds in conjugation and their geometrical configuration. A minimum of three double bonds in conjugation was required before reduction took place at the cathode, and as the number of conjugated bonds increased in the lipid compounds, the initial reduction wave took place at a lower half-wave potential.

Investigation of conjugated double bonds in triglycerides and in alkali-isomerized linolenic and arachidonic acids gave reduction waves the half-wave potentials of which were related to the number of double bonds in conjugation. In both basic and neutral solvents there was a minimum of three double bonds in conjugation necessary to obtain a reduction wave at the dropping mercury electrode. Ultraviolet absorption curves of the prolonged reduction of polyunsaturated conjugated fatty acids indicate a step-wise reduction of each end of the polyunsaturated conjugated double bonds.

In neutral solvent the log of the conjugated double bonds versus the half-wave potential (versus mercury pool anode) gave a linear equation, $E_{\frac{1}{2}} = 2.98 - 1.6 \log C$. A proposed mechanism for the step-wise reduction of conjugated lipids is presented and discussed.

INTRODUCTION

POLAROGRAPHIC INVESTIGATION of lipids had involved many difficulties which were caused by poor solubilities in common solvents employed in polarography. With mixtures of polar and nonpolar solvents and a suitable supporting electrolyte, organic polarography has been extended to lipid studies to include both triglyceride and organic peroxides (1,2), steroid (3), and some fat-soluble vitamins (4-6).

Although unsaturated hydrocarbons and conjugated aromatic polyene are known to be reduced in 5% to 25% aqueous protonic or nonaqueous solution (7-10), the application of this technique to conjugated vitamins has only recently received attention (11).

This paper investigates the conditions needed for the reduction of various naturally occurring conjugated lipids by the application of polarography and presents some evidence as to the mechanism of reduction of these conjugated lipids at the dropping mercury electrode. In this study a 10% aqueous dioxane containing tetrabutylammonium hydroxide or chloride, although not considered a protonic solvent, gave reproducible polarograms for conjugated fatty esters, triglycerides, sterols, β-carotene, and fat-soluble Vitamins A and D.

EXPERIMENTAL SECTION

Material and Equipment

All the conjugated triglycerides, tung, oiticica, parinarium, and isano oil, as well as 22% conjugated safflower oil, were obtained from the Pacific Vegetable Oil Company. Menhaden oil was furnished by the US Fish and Wildlife Service. The extracted oils were partly purified by passing a petroleum ether (bp 30-60C) or diethyl ether solution through activated alumina to remove any free fatty acids or minor peroxidic-containing material that might be present in the original oil.

Alpha and *beta* eleostearic acid as well as the methyl and ethyl esters were prepared from domestic tung oil by the procedure of Hoffman et al. (12). Ultraviolet absorption at 268 mμ for β-eleostearic and at 270 mμ for α-eleostearic acid showed purity of both acids to be greater than 97%. After activated alumina treatment the ultraviolet absorption maximum of tung oil was measured, and the percentage of eleostearic acid was calculated and found to be 73% (13). Parinarium Glabarinum had 33.8% parinaric acid, and isano oil had

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more than 50% "isanic" and "isanolic" acid (13).

All the conjugated fat-soluble vitamins studied were purchased from the Eastman Chemical Company and were used without further purifications. A comparison of infrared spectra with the known standard spectrum indicated a high chemical purity. Vitamins D_2 and D_3, all-*trans*-retinyl acetate, all-*trans*-retinol, 13-*cis*-retinol, and all-*trans*-retinal were soluble in 10% aqueous dioxane containing 0.1 M tetrabutylammonium chloride or hydroxide as a supporting electrolyte at 10^{-3} M concentrations or higher. Limited solubility of β-carotene made it necessary to run polarograms, starting at a maximum concentration of 10^{-4} M in tetrabutylammonium chloride, but in tetrabutylammonium hydroxide β-carotene was soluble at higher concentrations.

The methyl linoleate and linolenate were purchased from the Hormel Institute and were considered to be > 99% pure. The methyl arachidonate also from Hormel was > 90% pure. The methods of handling the highly purified unsaturated fat from Hormel were similar to those used for handling the conjugated fat-soluble vitamins. An IR spectrum, either of mineral oil mull or film, was a check on the identity of the sample. Open vials were placed into two amber bottles, one within the other, and an atmosphere between the sample and the two amber bottles was kept free of oxygen with nitrogen and kept stored at –20C. When a sample was necessary, the contents were brought to room temperature and opened and sampled under an atmosphere of nitrogen.

The tetrabutylammonium salts used as a supporting electrolyte were "polarographic grade," purchased from Southwestern Analytical Chemicals, Austin, Tex. Commercial dioxane was refluxed with sodium metal from 12 hr before distilling and was used as polarographic solvent. The mercury used for the dropping mercury electrode and anode was triple-distilled, C.P. grade, with maximum nonvolatile matter of 0.001%, and was purchased from Metalsalts Corporation, Hawthorne, N. J.

The polarograms were obtained on a Sargent Model XV from 0 to 3 volts with the polarizing voltage applied at a rate of 0.31 volts per minute. This voltage was applied to a jacketed polarographic cell maintained at a constant temperature of 25± 0.2C; the inner compartment contained a 1.1 cm^2 mercury anode. The resistance of the 10% aqueous dioxane containing either 0.1 M tetrabutylammonium hydroxide or the chloride, as measured by a conductivity bridge, was 9,800 and 23,000 ohms respectively. The conditions for measuring the capillary constant of the cathode were to maintain a mercury height of 720 mm, apply a voltage of –2.50 volts, and measure the drop-time, which was 1.58 seconds for the tetrabutylammonium hydroxide and 1.62 seconds for tetrabutylammonium chloride. The calculated capillary constant, $m^{2/3}t^{1/6}$ at –2.50 volts in tetrabutylammonium hydroxide, was 3.02 and in tetrabutylammonium chloride, 3.04 (14). The half-wave potentials and diffusion currents were measured from the polarograms by the procedure of geometrical rectangular coordinates.

Millicoulometry

The millicoulometric method of Weaver and Whitnach (15) for determining n-values for the conjugated lipids was modified to use a U-shaped electrolysis cell so that 0.3 ml of test solution could be reduced for various periods of time at a selected constant potential. During the two-hour electrolysis a small stream of solvent-saturated nitrogen was directed at the anode surface to renew constantly the surface of the anode to minimize the error attributable to the "depletion effect" of the conjugated lipids at the cathode (16).

From the expression of Faraday's law, $n = c/Fm$, where c is the number of coulombs passed during reduction, m the number of moles of conjugated lipid reduced by the passage of c, and F the 96,500 coulombs, n (the number of electrons per conjugated lipid transferred in electrolysis) can be calculated by experimentally determining m and c (15).

In determining the absorption spectra at the start and following the millicoulometry, eleostearic and parinarium triglycerides were diluted to ca. 10^{-5} M with 10% aqueous dioxane. The reference solution for the spectrophotometer was prepared in the same way as the original electrolytic solution. The absorption curves were obtained from 220 mμ to 350 mμ; no absorption was related to the purified dioxane or supporting electrolyte. The ultraviolet absorption curves were run on a Cary Model 14.

RESULTS AND DISCUSSION

Polyunsaturated Fatty Lipids

As an example of the results which were obtained from the polarograph, Figure 1 shows the polarograms of α and β-eleostearic acid in 10% dioxane containing 0.1 M tetrabutylammonium hydroxide. The A wave represents a

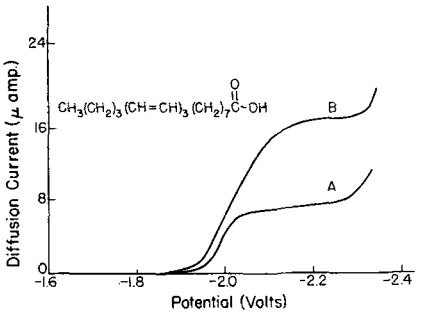

FIG. 1. Polarograms of eleostearic acids. A—0.50 micromole/ml α-eleostearic acid. B—1.25 micromole/ml β-eleostearic acid.

concentration of 0.50 micromole/ml α-eleostearic acid; curve B is 1.25 micromole/ml liter of β-eleostearic acid. When α and β-methyl eleostearate were run, the same half-wave potential was obtained. Also tung oil, which contained approximately 70% α-eleostearic acid, gave results similar to those mentioned above. Thus the ester, triglycerides, and free acid of eleostearic acid gave the same half-wave potential at the dropping mercury electrode. Since the only common group present in all the compounds was three double-bonds in conjugation, the reduction wave obtained for these compounds must be associated with the content of conjugation. Alpha- and β-methyl eleostearates, isolated by the procedure of Hoffman and co-workers (12), were measured at the same concentration and gave reduction waves that differed by 0.05 volts; this difference between the two isomeric forms was not large enough to differentiate them.

When other naturally occurring conjugated oils were measured, different half-wave potentials were obtained as well as a linear relationship between diffusion current and concentration tested (2.5 mg/ml). A straight-line curve of diffusion current vs. concentration for oiticica and isano oil was obtained when the diffusion current was measured at half-wave potential of –1.98 volts for oiticica and –1.80 volts for isano oil. Licanic acid, the principal acid in oiticica oil, differs from eleostearic acid in that it was a keto group in the four-position. Since isolated keto groups are not reduced with ease, the three double bonds in conjugation in licanic acid are responsible for the half-wave potential at –1.98 volts, which is similar for eleostearic acid. Isano oil, containing the two principal fatty acids, isanic and isanolic acid, which both have conjugated acetylenic bonds $\Delta^{9\text{-}11}$, gave reduction waves at –1.80 volts in basic solution. Since both acids have isolated terminal double bonds and the hydroxyl group on the 8th carbon in isanolic acid are not reduced under these conditions, the two acetylenic linkages in conjugation are probably reduced at a half-wave potential of –1.80 volts.

Another naturally occurring conjugated polyunsaturated fatty acid investigated was parinaric acid, found in the oil from Parinarium Gladarnium. The oil contains about 35% parinaric acid, which is octadeca 9:11:13:15 tetraenoic acid, and gave two reduction waves at $E_{\frac{1}{2}} = -1.60$, –2.20 volts. A linear relation ship between diffusion current and concentration was found for each reduction wave. The linearity of the calibration curves for oiticica and isano oil, as well as those for tung and parinarium oil, suggests that polyunsaturated conjugated fatty acids found in naturally occurring oils may be determined by polarography.

To determine the minimum number of double bonds that are reduced and to learn to what extent this procedure is applicable to methylenic interrupted double bonds found in fats and oils, samples of pure methyl linoleate, linolenate, and arachidonate were alkali-isomerized by the 21% KOH ethylene glycol procedure (17). Figure 2 shows the polarograms of the alkali-isomerized linolenic and arachidonic acids. Alkali-isomerized linolenic acid (curve A) gave a half-wave potential identical to eleostearic acid.

Alkali-isomerized arachidonic acid (curve B) had two reduction waves at half-wave potentials of –2.02 and –1.75 volts. The first

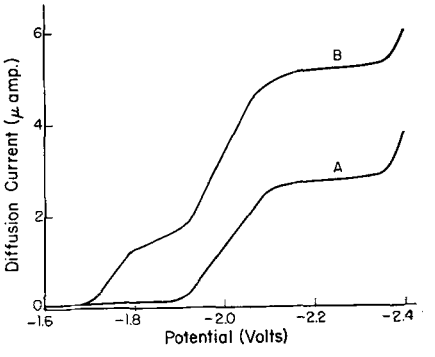

FIG. 2. Polarograms of alkali-isomerized linlenic (A) and arachidonic (B) acids. $E_{\frac{1}{2}} = -2.02$ volts for alkali-isomerized linolenic acid and $E_{\frac{1}{2}} = -1.75$ and –2.00 volts for alkali-isomerized arachidonic acid, obtained in 10% aqueous dioxane containing 0.1 M tetrabutylammonium hydroxide.

half-wave potential is similar to the first reduction wave of parinaric acid, which has four double bonds in conjugation whereas the second reduction wave had a half-wave potential almost identical to α or β-eleostearic acid. Although butadiene has been shown to give a reduction wave at $E_{\frac{1}{2}} = 2.63$ volts (10), alkali-isomerized methyl linoleate and 22% conjugated safflower oil were run without any indication of a reduction wave.

The results would indicate that the minimum number of double bonds in conjugation needed with fatty acids to obtain a reduction wave is three and that additional reduction waves appear at more positive potential as the number of double bonds increased. When a polyunsaturated marine oil like menhaden was alkali-isomerized, a polarogram was obtained that was similar to alkali-isomerized arachidonic acid. Another small reduction wave at a half-wave potential of –1.50 volts indicates that pentenoic and possibly hexenoic acids (18) are conjugated by the 21% KOH ethylene glycol procedure.

Evidence has been presented to show that this polarographic procedure could be applicable to the determination of conjugated polyunsaturated oil in which three or more double bonds are present. After alkali-isomerization the same principle applies for naturally occurring methylenic interrupted oil containing linolenic or higher unsaturated fatty acids.

Ultraviolet Absorption and Millicoulometry

To obtain more insight as to which double bond is reduced within the conjugated double bonds and the number of electrons transferred at the cathode, a millicoulometric study of eleostearic and parinarium oil was made. By following the ultraviolet absorption spectrum before and after prolonged reduction at the potential where the limiting current starts to level off, an indication of the step-wise reduction which takes place at the dropping mercury electrode can be measured. In the controlled reduction of methyl eleostearate at –2.20 volts in basic solution for two hours, the decrease in the absorbance at 270 mμ was accompanied by an increase in the absorbance at 234 mμ Since an increase in the absorbance at 234 mμ. is associated with triene conjugation and the peak at 234 mμ indicates diene conjugation, the triene reduction at the cathode probably takes place at either end of the conjugated system to produce $\Delta^{9\text{-}11}$ or $\Delta^{11\text{-}13}$ dienoic acid. Reduction of Δ^{11} double bond is also possible

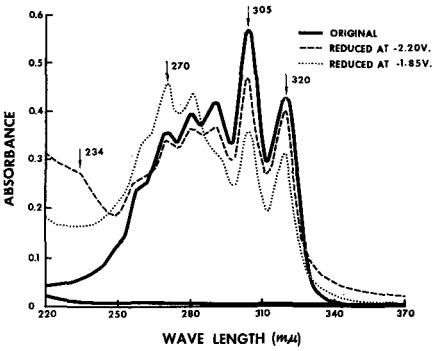

FIG. 3. Ultraviolet absorption spectrum of parinaric triglycerides:—original solution; - - - after one-and-a-half hours of controlled reduction at $E_{\frac{1}{2}} = -2.20$ V: .... after two-and-a-half hours of controlled reduction at $E_{\frac{1}{2}} = -1.85$ V.

with subsequent conjugation to give the same dienoic acids in alkali solution.

A similar experiment with parinarium oil is shown in Figure 3. The controlled polarographic reduction at –1.85 volts has the decided effect of decreasing the absorbance at 305 mμ, which is the predominant absorption maxima for four double bonds in conjugation, with a concurrent increase in absorption at 270 mμ. It should be pointed out that no absorption peak was found in 234 mμ when controlled polarographic reduction took place at –1.85 volts. When this sample is reduced at the potential of –2.20 volts, the absorption curve changed markedly with an increase in absorption at 234 mμ.

The changes in ultraviolet absorption curves of parinarium oil indicate that the polarographic reduction at a half-wave potential of –1.71 volts takes place with the reduction of four double bonds to three double bonds whereas at –2.01 volts reduction goes on further to the dienoic acid. These results, if analogous to methyl eleostearate, indicate that the outer double bonds Δ^9 and Δ^{15} in parinaric acid are reduced first to the triene and subsequently to the diene. It is proposed that the end-product of the complete polarographic reduction of parinaric triglycerides is either $\Delta^{9\text{-}11}$, $\Delta^{11\text{-}13}$, or $\Delta^{13\text{-}14}$ dienoic acid.

Additional evidence to support a step-wise reduction of one double bond in methyl eleostearate and parinarium oil can be shown from millicoulometry study. With a controlled reduction of eleostearate (15) at an applied potential of –2.20 volts, an experimental value of 2.2 was obtained for the number of electrons (n) transferred at the cathode. When

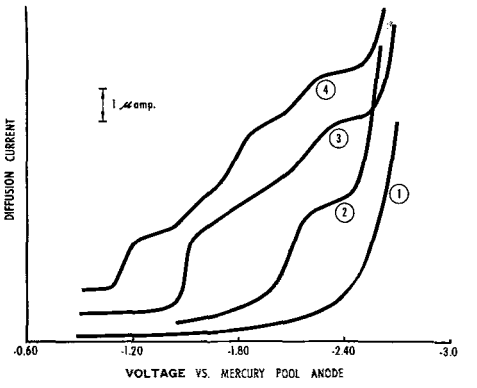

FIG. 4. (1) Solvent media, 10% aqueous dioxane containing 0.1 M tetrabutylammonium hydroxide. (2) Vitamin D_2, 5×10^{-4} M. (3) 13-*cis* Retinal, 5×10^{-4} M. (4) β-Carotene (all-*trans*), 5×10^{-4} M.

parinarium oil was reduced at an applied potential of –2.20 volts, the value for n was 2.2 for the reduction wave at $E_{\frac{1}{2}} = -1.71$ volts and 3.2 for the second reduction wave. The slightly higher value for the second reduction wave is in part caused by the presence of some eleostearate (Figure 4). The slightly higher values for n obtained above instead of the theoretical value of 2.0 for the first reduction wave in both conjugated fatty acids are inherent in the millicoulometry method, which measures a smaller integrated diffusion current than is present in the cell.

Reduction of Polyunsaturated Conjugated Vitamins

To extend these observations to other unsaturated lipids, attention was directed to the unsaturated fat-soluble vitamins. Figure 4 illustrates typical polarograms for a selected number of unsaturated conjugated vitamins with experimental conditions similar to those used on the conjugated polyunsaturated fatty acids. Curve 2 represents a 0.5 micromole/ml solution of Vitamin D, curve 3 the type of polarogram obtained from 13-*cis*-retinol, and curve 4 a 0.5 micromole/ml for β-carotene. The number of bonds in conjugation increases from three for Vitamin D_2 to five for Vitamin

TABLE I

Observed Half-Wave Potentials ($E_{1/2}$) for Conjugated Lipids

Double bonds[a]	$E_{1/2}$ (volts)	$\frac{id}{m^{2/3}t^{1/6}C}$ [b]	$E_{1/2}$ (volts)	$\frac{id}{m^{2/3}t^{1/6}C}$ [c]
3				
Vitamin D_2	—2.01	1.59	—2.25	1.50
Vitamin D_3	—2.01	1.66	—2.22	1.66
Eleostearic acid	—1.98	1.79	—2.20	1.57
Ergosterol[d]	—		—	
4				
Parinaric	—1.71	1.27	—1.87	1.55
acid	—2.03	2.17	—2.31	2.63
5				
Retinol	—1.53	1.59	—1.86	1.60
(all *trans*)	—1.77	1.00	—2.10	0.99
	—2.34	0.90	—2.58	0.92
Retinol	—1.50	1.59	—1.74	1.72
(13-*cis*)	—2.18	1.03	—2.37	1.19
Retinyl acetate	—1.53	1.59	—1.44	1.87
(all *trans*)	—1.71	1.00	—1.92	1.82
	—2.16	0.60	—2.59	1.07
Retinal	—0.78	0.74		
(all *trans*)	—1.14	0.94	—0.96	1.09
	—1.65	0.20	—1.26	1.32
	—2.19	1.20	—2.37	1.65
11				
β-Carotene	—1.14	0.84	—1.35	0.12
(all *trans*)	—1.50	0.40	—1.74	0.14
	—1.75	0.94	—1.96	0.14
	—2.10	0.74	—2.22	—

[a]Number of double bonds in conjugation.

[b]Half-wave potential with reference to mercury pool anode in 10% aqueous dioxane containing tetrabutylammonium hydroxide.

[c]Half-wave potential with reference to mercury pool anode in 10% aqueous dioxane containing tetrabutylammonium chloride.

[d]Not reduced under these experimental conditions.

A and finally 11 for β-carotene.

These selected polarograms show that, as the number of double bonds increases, the first reduction wave takes place at a more positive potential.

Table I tabulates a summary of the conjugated lipids material in which the structure and purity are known with some confidence. In basic and neutral solvents, the lipids containing three double bonds in conjugation have half-wave potentials within 0.2 volts and similar apparent diffusion coefficients. The isolated double bond in vitamin D_2 in the side chain which is attached to the cyclopentaphenanthrene is not involved in the reduction since the same half-wave potential was obtained for vitamin D_3. The absence of a reduction wave with ergosterol indicates that three double bonds are needed before the polarographic reduction of sterols. The apparent diffusion coefficient $i_d/m^{2/3}t^{1/6}C$ (where i_d, diffusion current in microamperes; m, rate of flow of mercury, mg/sec; t, time needed for one drop to fall in seconds; and c, concentration in millimoles/liter) indicates that the number of electrons transferred at the dropping mercury electrode is also two for three double bonds in conjugation.

It will be noted that most half-wave potentials ($E_{\frac{1}{2}}$) in neutral solution are more negative by about 0.2 of a volt from those in basic solution. The explanation for this difference is primarily related to the difference in the reference potential of the mercury pool anode. The difference between the potential of the half-cell of 0.1 M $OH^-/HgO/Hg$ and 0.1 M $Cl^-/Hg_2Cl_2/Hg$ cell is almost 0.2 of a volt. A second and smaller contribution to the $E_{\frac{1}{2}}$ difference is the IR drop across the polarographic cell. The resistance of the neutral solution is two-and-a-half times that of the basic solution; this amounts to about .03 of a volt when one microampere flows through the cell. These two factors seen to explain the almost consistently higher $E_{\frac{1}{2}}$ of about 0.2 of a volt in almost all the lipids studied in neutral solution.

Parinaric acid has two reduction waves. The first reduction wave at a $E_{\frac{1}{2}} = -1.71$ volts in basic solution and $E_{\frac{1}{2}} = -1.87$ volts in neutral solution is related to the reduction of four double bonds in conjugation; the second reduction wave is associated with the reduction of three double bonds in conjugation. The apparent diffusion constant for the first wave appears to involve the same number of electrons transferred as with three double bonds. The apparent diffusion coefficient for the second reduction wave for parinaric must be interpreted with some reservation because the parinaric acid contained some eleostearic acid which contributes to the higher diffusion current.

Under the experimental conditions used, all-*trans*-retinol shows three reduction waves at –1.53, –1.77, and –2.34 volts in basic solution whereas the 13-*cis*-retinol has only two reduction waves at –1.50 and –2.18 volts. When the geometrical configuration of one of the double bonds in conjugation at the end of the molecule is altered to *cis* configuration, the number of reduction waves is decreased. In neutral solution the introduction of a *cis* configuration causes the half-wave potential to become more positive, i.e., the 13-*cis*-retinol is easier to reduce.

Takahashi and Tachi (11), using acetonitrile-benzene mixture as a solvent containing 0.1 M tetrabutylammonium iodide as the supporting electrolyte, also observed three reduction waves. The first two were useful for their quantitative determination, but the third at –2.0 volts was ill-defined. Their results for retinol and retinyl acetate for the first reduction wave showed that two electrons are transferred at the cathode. The results presented would also indicate a two-electron transfer, when compared with eleostearic acid, because of the similarity in the apparent diffusion coefficient found in 10% aqueous dioxane in terabutylammonium chloride for all-*trans*-retinol, retinyl acetate, and 13-*cis*-retinol.

When the Vitamin A aldehyde was studied, the half-wave potential was altered when investigated in basic and neutral solutions. The first obvious difference was in the number of reduction waves; four reduction waves were obtained in basic solution but only three in neutral solution. Salah and Heyrovsky (19) did not show a reduction wave for Vitamin A but obtained two reduction waves for retinal in aqueous ethanol at pH 4 involving two electrons. This was related to the reduction of the aldehyde group to the alcohol and not to the conjugation. The half-wave potential of the first reduction wave in tetrabutylammonium chloride for all-*trans*-retinal is in close agreement with the value which Fields and Blout (20) obtained for 2,4,6,8,10 dodecapentaenal in 50% buffered dioxane, pH 5.05, when a correction is made for the differences in anodic electrode. The explanation they offered for the first of two waves was that the polyene aldehyde was reduced by a one-elec-

tron, possibly reversibly. Transfer followed by dimerization of the free radical.

Since no mention was made of the second reduction wave, it can be assumed that the aldehyde group was reduced. The reduction of all-*trans*-retinal observed at half-wave potentials of –2.19 and –2.37 volts in basic and neutral solution respectively was associated with the reduction of the aldehyde group. Evidence for reduction of the aldehyde group was obtained from polarograms of equal molar concentrations of all-*trans*-retinal and heptaldehyde (5×10^{-3} M) in both supporting electrolytes. The polarograms had half-wave potentials almost identical to those shown in Table I. The only significant difference was in the apparent diffusion coefficient, which increased by a factor of approximately 2.

All-*trans*-retinal in basic solution gave the lowest half-wave potential of all the conjugated lipids measured. The aldehyde group, even though its reduction takes place at –2.19 volts, may be involved by the formation of additional conjugation in the basic solution, which increased conjugation and resulted in a reduction taking place at a lower half-wave potential.

Since all-*trans* β-carotene increases the number of double bonds to 11, four reduction waves were obtained in both neutral and basic solution. The last reduction wave at $E_{\frac{1}{2}} =$ –2.22 volts was ill-defined and merged with the decomposition potential of the supporting electrolyte tetrabutylammonium chloride.

The difference in the apparent diffusion constant of β-carotene as compared with the other lipids studied shows a decrease in sensitivity by about one-half in basic solution and one-tenth in neutral solution. The decrease in sensitivity in neutral solution is partly because of limited solubility of β-carotene in 10% aqueous dioxane containing tetrabutylammoniun chloride. Calculated values for the apparent diffusion constant were made in the concentration range of 10^{-4} to 10^{-5} M neutral solution, but in the basic solution measurement were made from 10^{-3} to 10^{-4} M.

At present the only reasonable explanation for the apparent diffusion coefficient value in neutral solution at about one-tenth the value of the other lipids studied is that the diffusion coefficient for β-carotene would be approximately one-half that of vitamin A. Field and Blout (19) found the diffusion coefficient in 50% dioxane of 2,4,6,8,10 dodecapentaenal, as calculated from the Ilkovic equation, to be

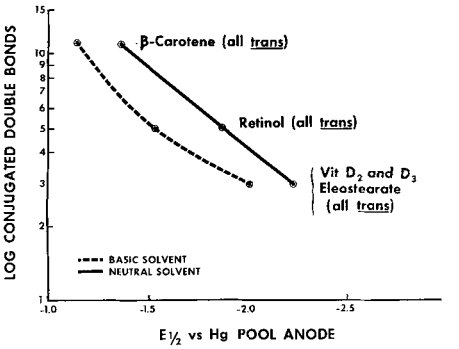

FIG. 5. Relationship between $E_{\frac{1}{2}}$ first reduction wave and log conjunction.
- - - - Basic solution, 10% aqueous dioxane containing 0.1 M tetrabutylammonium hydroxide.
—— Neutral solution, 10% aqueous dioxane containing 0.1 M tetrabutylammonium chloride.

4.2×10^{-6} cm²/sec. Each wave corresponded to a one-electron reduction.

This is similar to the value of 4.5×10^{-6} cm²/sec for β-carotene in the 60% benzene-acetonitrite mixture obtained by Takahaski and Tachi (11). If the first reduction wave of Field and Blout was a two-electron transfer, the diffusion coefficient of 2,4,6,8,10 dodecapentaenal, which is assumed to be similar in structure to Vitamin A, would be one-half that of β-carotene. Further experimental work is needed to determine the diffusion coefficient of Vitamin A in the solvent 10% aqueous dioxane containing tetrabutylammonium salts.

Reduction of Trans Configuration Lipids

In relating the half-wave potential of all-*trans* geometrical isomers of conjugated lipids with the log of the number of double bonds in conjugation, a plot is obtained as shown in Figure 5. Only compounds with known highest purity were used when the geometrical configuration of the various lipids was considered. In neutral solution the relationship between the log of the number of all-*trans* double bonds and half-wave potential is a has a bend, which indicates that the equation straight line. The equation of the line in neutral solution is $E_{\frac{1}{2}} = 2.98 - 1.6 \log C$, where $E_{\frac{1}{2}}$ is the half-wave potential vs. mercury anode in tetrabutylammonium chloride, 2.98 is the intercept on the X axis, 1.6 the slope of the line, and C is the number of double bonds in conjugation. In basic solution the curve for the curve would be a polynomial that could be obtained empirically in trying to fit an

equation to the plot. The explanation for this nonlinearity in the basic solution is believed to be related to the isomerization of the all-*trans* double bonds to *cis-trans;* this alters the half-wave potential of the first reduction wave.

The proposed mechanism for the first reduction wave is partly based on the previous work of Hoijtink et al. (7,8) with various conjugated hydrocarbons.

When conjugated lipids with three or more double bonds in conjugation are reduced at the dropping mercury electrode, an over-all two-electron transfer occurs. The reduction occurs in a step-wise sequence with R representing three or more double bonds in conjugation.

$R + e \rightleftarrows R^{\ominus}$ (reversible, potential-determining)

$R^{\ominus} + H_2O \rightarrow RH\cdot + OH^{\ominus}$ (irreversible, fast)

$RH\cdot + e \rightarrow RH^{\ominus}$ (irreversible, fast)

$RH^{\ominus} + H_2O \rightarrow RH_2 + OH^{\ominus}$

The first electron is reversible, as shown by the method of Tomes (21) which produces an anion; this first electron is also the potential-determining voltage of the first in a series of reduction steps. A water molecule reacts with the anion to form a free radical, which quickly reacts with another electron and gives an anion containing an additional hydrogen atom. The anion then reacts with another water molecule to saturate one of the double bonds in conjugation. It is possible that this series of reactions repeats itself with more than three double bonds until a diene is formed. If the results plotted in Figure 5 are extrapolated to the point where all *trans* conjugated diene would be reduced, a voltage of –2.52 in neutral solution would be needed. This potential was not observed under these experimental conditions.

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Inhibition by Cyclopropene Fatty Acids of the Desaturation of Stearic Acid in Hen Liver[1]

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ABSTRACT

The mechanism of the hardening of body fats of animals by dietary lipids which contain cyclopropene fatty acids has been studied. Dietary methyl sterculate increased the stearic acid content of egg yolk lipid and decreased the activity of the stearic acid desaturase system of hen liver. The cyclopropene fatty acids were specific inhibitors of the stearic acid desaturase system of hen livers since other fatty acids, including two possible metabolites of sterculic acid, failed to inhibit the system at equivalent concentrations. Sterculic acid was a more effective inhibitor of the system than malvalic acid. Kinetic studies have shown that the inhibition is irreversible. Apparent kinetic constants were determined for the system.

The results support the hypotheses that cyclopropene fatty acids inactivate an essential component of the desaturase system, probably by combination with -SH groups, and that this inhibition causes many of the effects of dietary cyclopropene fatty acids, including permeability disorders of eggs.

INTRODUCTION

STERCULIC AND MALVALIC ACIDS are two cyclopropene fatty acids present in plants of the order Malvales (1,2), of which cotton is the commercially important member. Early observations had shown that pigs, cows, and hens fed cottonseed meal or oil yielded respectively a hard lard (3,4,5), a sticky butter with a high melting-point (6,7), and pasty yolks in the eggs subsequently laid by hens (8). Subsequent work showed that the feeding of cyclopropene fatty acids to farm livestock produced in them an increase in the proportion of stearic acid and a complementary decrease in the proportion of oleic acid present, in body fat (9), butterfat (10), and egg yolk fat (11,12) respectively.

In hen eggs there is also an increase in permeability of the membranes between the yolk and the white, leading to the pink white disorder that has been shown to be caused specifically by the cyclopropene ring of sterculic and malvalic acids (13,14,15).

An explanation for the biological activity of the cyclopropene compounds has been suggested by Kircher (16) who, by using methyl sterculate and mercaptans, demonstrated that a reaction between the thiol group and the cyclopropene double bond can occur. Accordingly he postulated that similar reactions may occur with available -SH groups of the "physiologically active proteins." This view is supported by the results of Ory and Altschul (17), who showed that the inhibition of the lipase of castor bean by cyclopropene fatty acids was partially countered by adding cysteine to the system.

The effect of the cyclopropene fatty acids on the desaturation of stearic to oleic acid has been investigated by Reiser and Raju (18), who reported that the desaturation of stearic acid in rats in vivo was inhibited by feeding *Sterculia foetida* oil. Johnson et al. (19), working with preparations of hen liver (20,21), demonstrated that pure sterculic or malvalic acids also inhibited the stearic acid desaturase system in vitro. Raju and Reiser (22) have suggested that cyclopropene fatty acids inhibit the comparable desaturase system of rat liver through an irreversible binding of the sulfhydryl groups as previously indicated.

The present report describes the effects in hens of dietary methyl sterculate on the production of egg disorders, the fatty acid composition of egg yolk lipid, and the activity of the liver stearic acid desaturase system. Sterculic, malvalic, and linoleic acids were also added in vitro to liver preparations from control hens to study their effects on the kinetics of the stearic acid desaturase system. In addition, two products derived from sterculic acid, possible metabolites, were used; one was dihydrosterculic acid, the other a mixture consisting of 9-methyleneoctadec-10-enoic acid and its isomer 10-methyleneoctadec-8-enoic acid. This mixture was regarded as one functional entity and will hence forth be referred to as

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"diene acid." This is a major product of opening the cyclopropene ring by any of several different methods, e.g., heating (23), treatment with silver nitrate-silica gel (24), or adsorption on alumina (25). The biological effects of dietary cyclopropene compounds will be discussed in relation to the inhibition of the stearic acid desaturase system.

EXPERIMENTAL SECTION

Methyl sterculate, methyl malvalate, methyl linoleate, and the methyl esters of the diene acid were prepared by the methods of Kircher (16), Fogerty et al. (26), Johnson and Ali (27), and Johnson et al. (24) respectively. Methyl dihydrosterculate was prepared by hydrogenation of methyl sterculate and isolation of the cyclopropane ester by preparative gas-liquid chromatography (GLC). Stearic acid was obtained from the Unilever Research Department, Port Sunlight, England, and stearic acid-1-^{14}C (specific activity 32.1 mc/mM) from the Radiochemical Centre, Amersham, England.

The purity of the dihydrosterculic acid, linoleic acid, diene acid, and stearic acid was better than 99%, as determined by GLC of their methyl esters. The purity of the methyl sterculate and malvalate was established by several methods. Thus a purity of at least 98% was indicated by adding methyl palmitate as an internal standard and analyzing the mixture by GLC after any of the following procedures: hydrogenation (24); treatment with silver nitrate on silica gel, followed by hydrogenation (24); treatment with methyl mercaptan (28). In addition, thin-layer and reversed-phase column chromatography (26) and also IR, NMR, UV, and mass spectra showed no departures from expected patterns for the methyl esters of sterculic or malvalic acids. By gas-liquid radio-chromatography it was established that the radiochemical purity of the stearic acid-1-^{14}C was at least 99%.

Prior to each incubation experiment the methyl esters of sterculic, malvalic, dihydrosterculic, linoleic, and diene acids were chromatographed on Florisil (29) and saponified at 37C overnight, under nitrogen, with a slight excess of 0.125N KOH in 95% methanol. Thereafter the solvent was evaporated with a stream of nitrogen, and the potassium salts of the acids were dissolved in 2.5% aqueous bovine serum albumin (K&K Laboratories, Calif.). The potassium stearate and potassium stearate-1-^{14}C were prepared from their acids, and the required dilutions were made with 2.5% aqueous bovine serum albumin.

The livers used were from bantam and normal White Leghorn hens which were maintained on a commercial laying ration. Three of the bantam hens were given daily doses of pure methyl sterculate (2.8, 6.9, and 13.9 mg/kg/day) in gelatin capsules for one year before killing. Liver supernatants, containing soluble enzymes and microsomes, were prepared and incubated in media as previously described (19).

Incubations were for 30 min except when the effect of time was being studied. Each incubation mixture contained 3.46 μc of potassium stearate-1-^{14}C and, except where specified, the concentration of stearic acid was 0.1 mM. After incubation 36 ml of chloroform-methanol (1:2, v/v) were added, and the lipid was extracted from the incubation mixture by using a modified Bligh and Dyer method (30), in which blending was omitted and a solution of 3% zinc acetate was added prior to filtration.

One-half of the extracted lipid was saponified under reflux with 0.04N KOH in 90% methanol for 1.5 hr, and the fatty acids were isolated. The methyl esters of the acids were prepared with boron trifluoride-methanol (31) and purified on Florisil (29). The methyl esters were chromatographed on 10 g of 25% silver nitrate on silica gel (Adsorbosil CABN 1420; Applied Science Laboratories, College Station, Pa.) in a column with four stages of diminution in diameter (32). The saturated methyl esters were quantitatively eluted from this column with 130 ml of benzene-hexane (30:70, v/v), and the unsaturated methyl esters with 100 ml of diethyl ether.

Radio-chromatography by GLC (33) and thin-layer chromatography on plates impregnated with silver nitrate (34) showed that the only radioactive components in the saturated fraction and in the unsaturated fraction were respectively methyl stearate and methyl oleate.

At each stage in the procedures the radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3324 by using conventional scintillation fluids. The over-all recovery of radioactivity was 85-90%.

Enzymic activity of the stearic acid desaturase system was expressed as percentage conversion which was calculated from the ratio of the count found in methyl oleate to the sum of the counts found in methyl oleate and in methyl stearate respectively. The amount of oleic acid which was formed was calculated from percentage conversion and the specific ac-

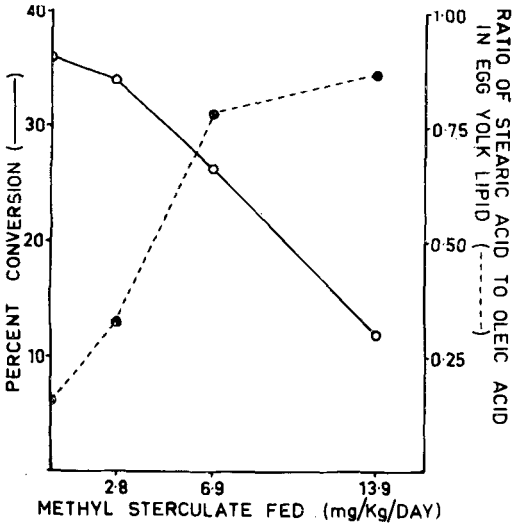

FIG. 1. Effect in hens of dietary methyl sterculate on the percentage conversion of stearic to oleic acid by the liver desaturase system and on the ratio of stearic acid to oleic acid in the egg yolk lipid.

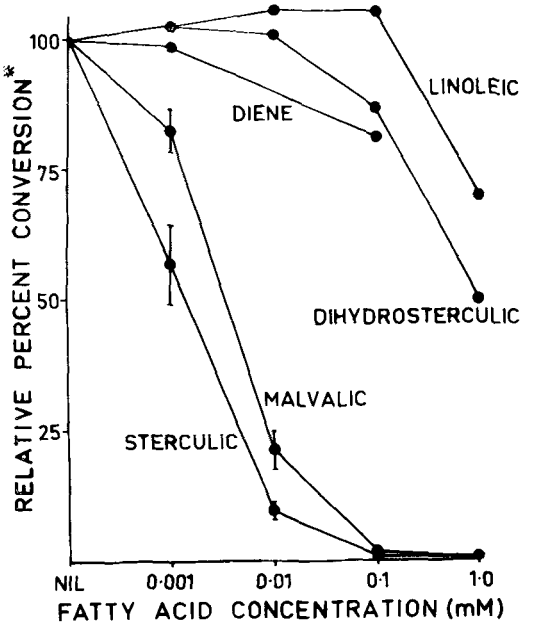

FIG. 2. Effect of fatty acids on the conversion of stearic acid to oleic acid by the hen liver desaturase system.

*The ^{13}C-oleate content relative to that in control flasks (100%) which had no fatty acid added other than stearic acid.

I= standard error of mean.

tivity of the stearic acid in each of the media at the commencement of the incubation. Lineweaver-Burk plots (35) were calculated as lines of best fit, and apparent K_m values were calculated by the method of Wilkinson (36).

The yolk lipid from the eggs of control and sterculate-fed hens was extracted (30), and the fatty acid composition was determined by GLC analysis of the methyl esters by using methods previously described (33). Eggs were also stored at 20C for at least six months, and the extent of the pink white disorder was visually assessed.

RESULTS AND DISCUSSION

The results of the feeding experiments on laying hens showed clearly that the addition of pure methyl sterculate to the hen diets increased the ratio of stearic to oleic acid in the yolk lipid of their eggs (Fig. 1). There was an inverse relationship between the dietary level of methyl sterculate and the desaturase activity of the liver enzyme system in converting stearic to oleic acid. Accordingly it appears that sterculic acid inhibits the enzyme system, thereby leading to increased stearic acid content and hardness of egg yolk lipid.

Eggs from hens fed on 6.9 and 13.9 mg/kg/day methyl sterculate showed typical permeability disorders, such as pink whites, but at a level of 2.8 mg/kg/day these defects were not apparent even after prolonged storage. However, at this lower level, an increase in the stearic acid concentration of the egg yolk was found (Fig. 1). It is therefore apparent that the increase in stearic acid content is a more sensitive index of the result of feeding cyclopropenoids than are permeability changes in the eggs.

The effect of various concentrations of sterculic, malvalic, and other fatty acids on liver enzyme systems which were prepared from control hens is shown in Fig. 2. Sterculic acid and malvalic acid both inhibited desaturation almost entirely when their concentrations were equimolar (0.1 mM) with that of the substrate stearic acid. At lower levels of each cyclopropene fatty acid the degree of inhibition was directly related to its concentration. Sterculic acid was a more effective inhibitor of the enzyme system than malvalic acid at concentrations of either 0.001 mM or 0.01 mM; the observed differences were statistically significant ($P<0.05$).

In contrast to the cyclopropene fatty acids which were tested, dihydrosterculic acid, the diene acid, and linoleic acid had no inhibitory

effect unless their concentrations were equal to or greater than that of the substrate. From these observations it was clear that the stearic acid desaturase system is specifically inhibited by cyclopropene fatty acids at low concentrations.

To further understanding of the nature of this inhibition, the first step was to initiate a series of experiments in which the substrate concentration was varied, both in the absence and in the presence of sterculic acid, malvalic acid, linoleic acid, or dihydrosterculic acid respectively. The activity of the stearic acid desaturase system in the liver showed some variation from hen to hen, indicated by the apparent K_m values which were obtained from separate livers, viz., 0.95, 2.65, 4.26, 4.53, and 7.28 mM. However in each case the cyclopropene fatty acids inhibited the system, and sterculic acid was always a more effective inhibitor than malvalic. From kinetic data relating to concentrations of either 0.1 mM linoleic acid or 0.001 mM dihydrosterculic acid, reciprocal plots were obtained which were coincident with those calculated for their respective controls, thus showing that, over the range of concentrations which were tested, these acids do not inhibit the activity of the desaturase system in vitro. Lineweaver-Burk plots of data from a typical experiment, which involved a single liver only, are shown in Fig. 3. Since these plots yielded a common intercept on the y-axis, the inhibition could be either competitive reversible or irreversible.

In order to differentiate between these two possibilities, further experiments had to be conducted. Accordingly the effect of incubating the enzyme system for several periods of time with a fixed initial concentration (0.1 mM) of substrate stearic acid and varying concentrations of the cyclopropene acids was studied (Fig. 4). Since no additional product had formed after 15 min of incubation with sterculic acid at a concentration of 0.1 or 0.01 mM, or with malvalic acid at 0.1 mM, it was concluded that inhibition of the system by the cyclopropene acids was irreversible.

Initial formation of oleic acid was observed and reflects competition for the enzyme system between the substrate and the irreversible inhibitor. However, because the initial substrate concentration was constant, the amount of oleic acid formed would depend on the inhibitor concentration. At 0.001 mM concentration a significant amount of enzyme activity remained hence this concentration of the cyclopropene was insufficient to inactivate entirely

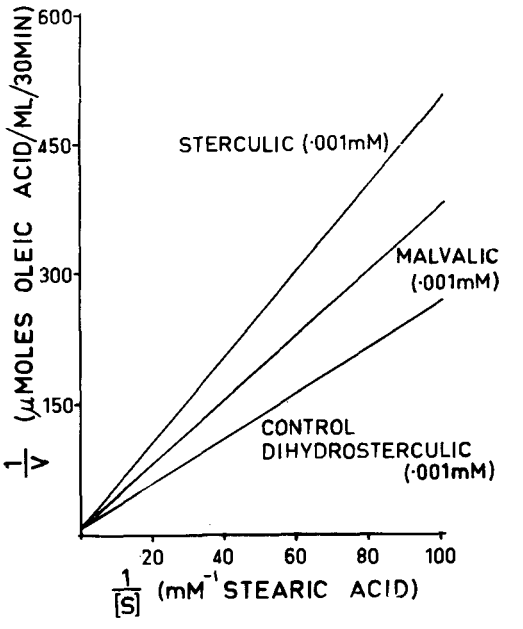

FIG. 3. Lineweaver-Burk plot for the stearic acid desaturase system in the presence and absence of cyclopropene and other fatty acids.

the enzyme system. In the absence of inhibitor the rate of formation of product was constant for at least 30 min.

Irreversibility, or at least an extremely strong affinity of the inhibitor for the enzyme relative to the substrate, was verified by prior incubation of the desaturase system with 0.01 mM sterculic acid, followed 5 min later by the

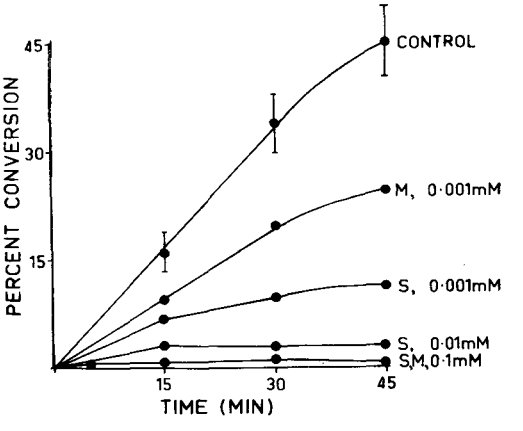

FIG. 4. Effect of time of incubation and concentration of sterculic (S) and malvalic (M) acid on the percentage conversion of stearic to oleic acid by the hen liver desaturase system.

I = standard error of mean.

addition of stearic acid at levels up to 100 times that of the inhibitor. In all cases inhibition was complete, showing that the excess substrate was unable to displace the inhibitor from the enzyme. In the absence of sterculic acid, prior incubation did not affect the conversion rate of appropriate controls.

At present the mechanism whereby the cyclopropene fatty acids specifically and irreversibly inhibit the stearic acid desaturase system can only be the subject of speculation. It is possible that the inhibition is attributable to inactivation of -SH groups of the system by combination with the cyclopropene at the double bond of the ring (16,17,22). Indeed, the stearic acid desaturase system may be particularly susceptible to the inactivation of its -SH groups by the cyclopropene fatty acids.

Shenstone and Vickery (15) have shown that the rate of onset of some of the defects in eggs from hens which were fed sterculic acid is about twice that in eggs from hens fed malvalic acid. The present work shows that sterculic acid is a more effective inhibitor than malvalic acid, and this could be because of steric differences in regard to the location of the -SH groups of the active site of the appropriate enzyme.

Inhibition of the desaturase system by the cyclopropene fatty acids could explain many of their deleterious effects, such as the pasty yolk effect. The work of March et al. (37) shows that in chicken erythrocytes there is an inverse relationship between their stearic acid content and the stability of these cells to hemolysis. If this relationship is applicable to all membrane systems, inhibition of the stearic desaturase system could lead to a high stearic acid content of membranes both within and around the yolk. Consequent increases in the permeability of such membranes would result in the development of the pink white and related disorders.

ACKNOWLEDGMENTS

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Biosynthesis of Phospholipids in Subcellular Particles from Cultured Cells of Human Tissue

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ABSTRACT

A time study of the incorporation of $^{32}P_i$ into the phospholipids of HeLa, KB, human heart, and liver tissue-culture cell lines has been carried out. The incorporation of $^{32}P_i$ at various time-intervals into the phospholipids of nuclei, mitochondria, and microsomes of HeLa and KB cells was investigated. The labeling of the isotope into the phospholipids was divided into three groups.

The first had two components: phosphatidyl inositol and polyglycerol phosphatides, which showed the greatest incorporation of the isotope as demonstrated in the specific activity values and the percentage of total radioactivity after 15 to 30 minutes of incubation. A second group was composed of the major phospholipids of all tissue-culture cell lines studied, phosphatidyl choline, and phosphatidyl ethanolamine. At first, there was a delayed labeling of these phospholipids; however, after one hour of incubation, a rapid increase was shown in the incorporation of $^{32}P_i$. A third group of lipids containing sphingomyelin and phosphatidyl serine demonstrated low specific activity values.

The phospholipids of the subcellular fractions, nuclei, mitochondria, and microsomes, had a high degree of incorporation of the isotope into the individual phospholipids and probably represented an active process in the membranes of these cellular units or a renewal of the biological membrane structures.

INTRODUCTION

PHOSPHOLIPIDS are found in membranes (8) of mitochondria, nuclei, and microsomes of mammalian tissues (13,21,22). Liver mitochondria of rats are composed of about 21-28% phospholipids on a dry-weight basis; the phospholipids account for about 90% of the total lipids present (21,22). The $^{32}P_i$ (inorganic phosphate) incorporation of phospholipids has been studied in vivo in animal tissues, but little is known about phospholipid synthesis in tissue-culture cells. This report presents the results of $^{32}P_i$ incorporation into the individual phospholipids of various tissue-culture cell lines and in the subcellular fractions, nuclei, mitochondria, and microsomes, of HeLa cells and KB cells.

MATERIALS AND METHODS

Cell Growth

Four kinds of heteroploid serial cultures were originally obtained from Microbiological Associates, Bethesda, Md.; HeLa (Gey), KB (Eagle), Human adult heart (Girardi), and liver (Chang). The cells were grown as monolayers in 1,000-ml bottles and were overlaid with 50 ml of growth medium, which was made up of Hank's salt solution (23) containing 10% of human serum, 1.0% of glucose, 2% of yeast extract, 1.4% of $NaHCO_3$, according to the procedure of Syverton et al. (23). To the solution was added an amount of penicillin and streptomycin to yield a final concentration of 100 units and 100 μg per ml respectively. An antifungal agent (mycostatin 50 μg per ml) was added routinely once a month but was absent when the cells were planted for experimental studies.

The cells were grown in a stationary state at 37C, and the growth medium was changed every other day. The inoculum of cells per each bottle was from 0.5 to 1.0×10^6, and, by the time of harvesting, approximately 5 to 10×10^6 cells were in each bottle.

Incubation with Radioactive Inorganic Phosphate ($^{32}P_i$)

Radioactive $^{32}P_j$ ($NaH_2\ ^{32}PO_4$) was obtained from the Oak Ridge National Laboratories and was diluted with GKN solution (Hank's solution: 1.0% of glucose, 0.8% of NaCl, and 0.04% of KCl) to a concentration of 30 mc/ml. The $^{32}P_i$-GKN solution was sterilized before it was added to the medium. On the day before the harvesting of the cells the medium was replaced by 45 ml of fresh growth-medium. Five ml of $^{32}P_i$ containing 150 μc were added to each culture bottle, and the cells were harvested at various time-intervals from 15 to 240 min after administration of the isotope. The final incubation medium

[1] Part of a thesis submitted to the Graduate School of the University of North Dakota in partial fulfillment for the degree of Doctor of Philosophy. Present address: Research Associate, Rockefeller University, New York. N. Y.

contained 3 μc of $^{32}P_i$/ml. When the cells were ready for harvesting, the growth medium was poured off and the cells were washed twice with 10 ml of ice-cold GKN solution. The cells were scraped, pooled, and suspended in an ice-cold 0.25 M sucrose solution containing 0.00018 M $CaCl_2$. The suspension was centrifuged at 700 $\times g$ for 10 min. The sediment was resuspended in sucrose-$CaCl_2$ solution and homogenized with a Teflon pestle for one minute in an Elvehjem tissue grinder.

Cell Fractionation and Lipid Extraction

The homogenate was brought to 9 ml with the sucrose-$CaCl_2$ solution. The suspension was centrifuged at 450 $\times g$ for 10 min. The sediment was resuspended in sucrose-$CaCl_2$ solution, and homogenization and centrifugation were repeated twice. The final sediment was considered a nuclear fraction. The pooled supernatant fractions were centrifuged at 10,000xg for 15 min in a Servall Automatic Superspeed Refrigerated Centrifuge, Type RC-2, to sediment the mitochondria. The remaining supernatant was, in turn, centrifuged at 80,000 $\times g$ for 90 min in a Spinco (Beckman) Model L, Preparative Ultracentrifuge, to sediment the microsomes.

The lipids of homogenate for the whole cell studies on the subcellular fraction, nuclei, mitochondria, and microsomes were extracted with 95% ethyl alcohol as described (25). Individual phospholipids were separated by silicic acid-impregnated glass paper chromatography and were identified by the method as described (25).

Radioactive and Quantitative Determination of Phospholipids

The spots of individual phospholipids from the chromatograms were removed, and radioactivity was determined with FD 1 inch thin-window flow counter on a SC-100 Multi/Matic Sample Changer (Tracer-lab Inc.), and each sample was corrected for decay.

After the determination of radioactivity the chromatogram spots were placed into 15 x 125-mm Pyrex test tubes, and the lipids were eluted with 5 ml of 3 N methanolic HCl for 50 min in a water bath at 63C. The elution was repeated two times with 5 ml of 2 N methanolic HCl. Lipid phosphorus was determined by a colorimetric method of Shin (20) and Bartlett (3). From the phosphorus and radioactivity analysis the specific activity (counts per minute/mg of phosphorus) was calculated.

Protein Determination

The dried residue after lipid extraction was treated with 5 ml of cold trichloroacetic acid (TCA) to extract the acid-soluble phosphorus fraction (19). Total nucleic acids were then extracted from the remaining residue with 5 ml of 5% TCA for 30 min at 90C, according to the procedure of Logan et al. (14). Protein-nitrogen of the residue after extraction of the lipid and nucleic acids was determined by means of the ultramicro-Kjeldahl method (17). From the radioactivity and protein-nitrogen data the counts/minute/mg of protein nitrogen were calculated so that a comparison between the different cell lines could be made.

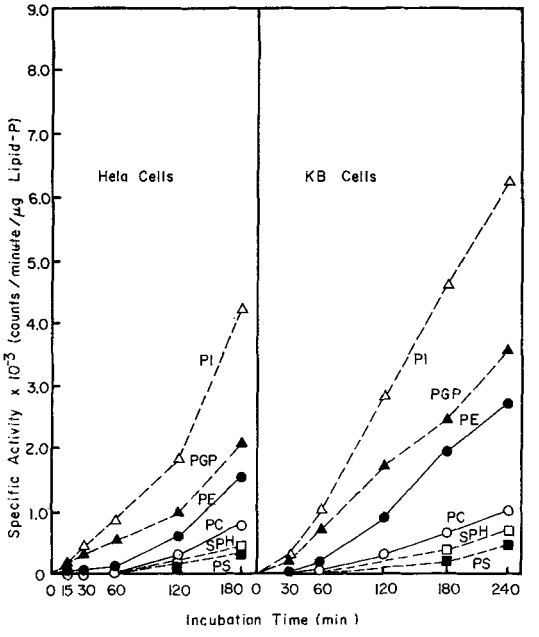

FIG. 1. Specific activity time-curves of individual phopholipids of HeLa and KB cells. Nine-day old HeLa and KB cells, grown as monolayer in growth medium containing 10% of human serum, were incubated with $^{32}P_i$ (3 μc/ml). One bottle of cells was used for each designated incubation time-interval. Abbreviations are: PI, phosphatidyl inositol; PGP, polyglycerol phosphatide; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; SPH, sphingomyelin; and PS, phosphatidyl serine.

RESULTS AND DISCUSSION

Fig. 1 illustrates the specific activities of the individual phospholipids of HeLa and KB cells as a function of time after incubation with inorganic $^{32}P_i$. The greatest incorporation of the isotope, as indicated in the specific activity values, occurred in phosphatidyl inositol, followed by polyglycerol phosphatide (or cardiolipin), phosphatidyl ethanolamine, phosphatidyl

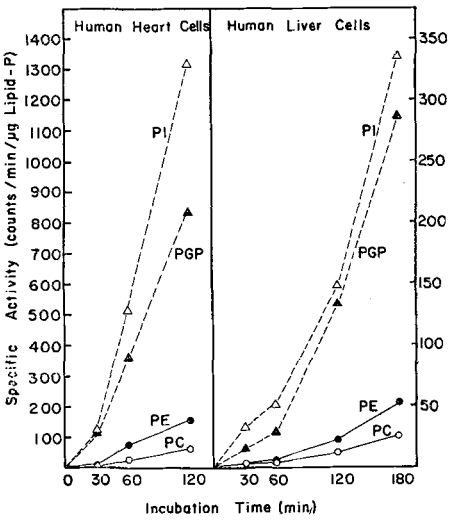

FIG. 2. Specific activity time-curves of individual phospholipids of human heart and liver cells. Six-day old cells of human heart and liver cells, grown as monolayer in growth medium containing 10% of calf and human serum respectively. The cells were incubated with $^{32}P_i$ (3 µc/ml) in growth media. Two bottles of cells were pooled for each designated incubation time-interval. Abbreviations are given in Fig. 1.

choline, sphingomyelin, and phosphatidyl serine. A time study of the incorporation of inorganic $^{32}P_i$ into the individual phospholipids of human heart and liver is presented in Fig. 2. The greatest incorporation of the isotope occurred in the phosphatidyl inositol and polyglycerol phosphatide in these two fractions of human heart and liver cells. It is apparent from these data that there is a difference of incorporation of the isotope between the different tissue-culture cell lines; however the pattern follows the same order. Lower specific activities were observed in the phospholipids which were isolated from human liver as compared with the other cell lines.

The labeling of the isotope into the phospholipids can be divided into three groups. The first has two components, phosphatidyl inositol and polyglycerol phosphatide, which show a high incorporation of the isotope, as demonstrated in the specific activity values after 15 to 30 min of incubation. A second group is composed of the major phospholipids of all tissue-culture cell lines studied, phosphatidyl choline, and phosphatidyl ethanolamine. At first there was a delayed labeling of these phospholipids; however after one hour of incubation a rapid increase was shown in the incorporation of $^{32}P_i$. A third group of lipids, containing sphingomyelin and phosphatidyl serine, demonstrated low specific activity values. This pattern of $^{32}P_i$ labeling of phospholipids is similar to that found in phagocytotic leukocytes (11) and in rat intestinal mucosa (24).

The increased incorporation of $^{32}P_i$ into polyglycerol phosphatide is similar to that observed in plants (18). Garbus et al. (6) have demonstrated that isolated mitochondria from rat liver and kidney have the greatest incorporation of inorganic $^{32}P_i$ into phosphatidic acid and phosphatidyl inositol during the first 18 min of incubation of the isotope as compared with phosphatidyl choline and phosphatidyl ethanolamine.

Zilversmit et al. (27) have shown by isotope labeling that it is possible to ascertain the conversion of a compound into another by determining the time uptake of the isotope and comparing the specific activity of the precursor and product. The precursor must have a higher specific activity than the product (27). Marinetti et al. (15) were able to demonstrate in $^{32}P_i$ incorporation studies of the whole liver of the rat that specific activities of phosphatidyl ethanolamine reached a peak before phosphatidyl choline and subsequently fell below phosphatidyl choline. This would indicate in whole liver that phosphatidyl ethanolamine may be a precursor of phosphatidyl choline or represents some interconversion of phospholipids. Further to substantiate this pathway Artom (1) has demonstrated in rat liver homogenates that the methylation of phosphatidyl monomethylethanolamine and phosphatidyl dimethylethanolamine form phosphatidyl choline.

Another pathway for the synthesis of phosphatidyl choline in mammalian tissue, demonstrated by Kennedy (12), involves cytidine diphosphate choline and diglycerides. Borkenhagen et al. (4) have reported enzymatic conversion of phosphatidyl serine to phosphatidyl ethanolamine in rat liver microsomes. It is apparent from the specific activity time curves in Fig. 1 and 2 that phosphatidyl ethanolamine may be converted to phosphatidyl choline since higher specific activity values were observed throughout the time study for phosphatidyl ethanolamine.

McCarl and Triebold (16) have reported the uptake of $^{32}P_i$ into phospholipids of HeLa, chicken embryo endothelium, bovine lymphosarcoma, and monkey heart cells. However the percentage of radioactivity was reported for the individual phospholipids for only the last

three cell lines. The percentage of radioactivity does not represent the same measurements as specific activity. Specific activity is radioactivity of phospholipids, divided by the pool quantity of phospholipids (counts/minute/μg of lipid P), whereas the percentage of radioactivity is only the distribution of the radioactivity.

A time course of distribution of radioactivity (percentage of total) for the individual phospholipids of HeLa cells, incubated for various intervals ranging from 15 to 1,200 min, is reported in Fig. 3. The greatest distribution of radioactivity was observed in phosphatidyl inositol fraction after 15 min of incubation, and a peak was observed in 30 min, which was followed by a decline. There is a progressive increase in the percentage of radioactivity in phosphatidyl choline and phosphatidyl ethanolamine fractions from 30 to 1,200 min to incubation.

The phosphatidyl choline fraction accounts for 56% of total radioactivity after 1,200 min of incubation. This observation is similar to that noted by McCarl and Triebold (16), who have shown an increase of percentage of total radioactivity for phosphatidyl choline from lymphosarcoma, monkey heart and chicken embryo endothelium cells incubated from 5 to 30 hrs. The incorporation of the isotope into the phospholipids has been related to the protein concentration, i.e., (counts/minute/μg of protein nitrogen) of HeLa and KB cell lines. The expression of isotopic data as a ratio to protein nitrogen will enable a comparison between two different cell lines. McCarl and Triebold (16), by using this technique, were able to observe a different incorporation $^{32}P_i$ into phospholipids for each cell line studied. The KB cells have a greater incorporation of the isotope into the lipids than HeLa cells. The pattern of incorporation of the isotope into the individual phospholipids is similar to the specific activity values.

The incorporation of $^{32}P_i$ into the individual phospholipids of nuclei, mitochondria, and microsomes of HeLa and KB cells is reported in Figs. 4 and 5. It is apparent from the data that the greatest incorporation of the isotope occurs in phosphatidyl inositol in all the subcellular fractions. The pattern of incorporation of the isotope into other phospholipids is similar for each of the other subcellular fractions. The specific activities of phosphatidyl ethanolamine is greater than phosphatidyl choline in all the subcellular fractions. Lecithin biosynthesis in microsomes is known to occur

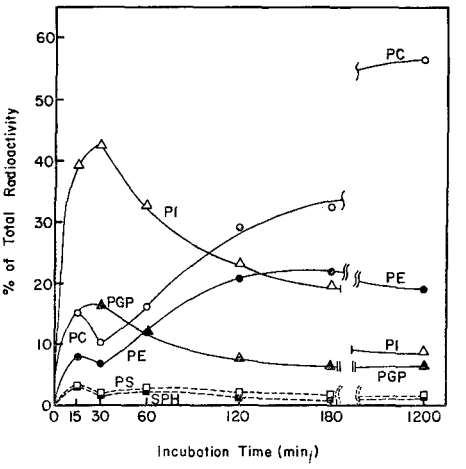

FIG. 3. A time course of distribution of radioactivity (percentage of total) for the individual phospholipids of HeLa cells. Nine-day old HeLa cells, grown as monolayer in growth medium containing 10% of human serum, were incubated with $^{32}P_i$ (3 μc/ml). One bottle of cells was used for each designated incubated time-interval. Abbreviations are given in Fig. 1.

by two pathways, one involving cytidine diphosphocholine and D-1,2-diglyceride (10) and the second occurring from repeated transfer of methyl groups from adenosyl methionine to phosphatidyl ethanolamine (2).

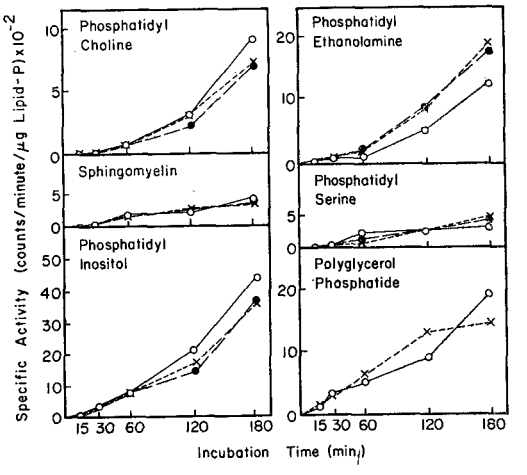

FIG. 4. Specific activity time-curves of individual phospholipids in the subcellular fractions of HeLa cells. Nine-day old HeLa cells, grown as monolayer in growth medium containing 10% human serum, were incubated with $^{32}P_i$ (3 μc/ml). Three bottles of cells were pooled for each designated time-interval: (x—x), nuclear; (o—o), mitochondria; and (●—●), microsomal fraction.

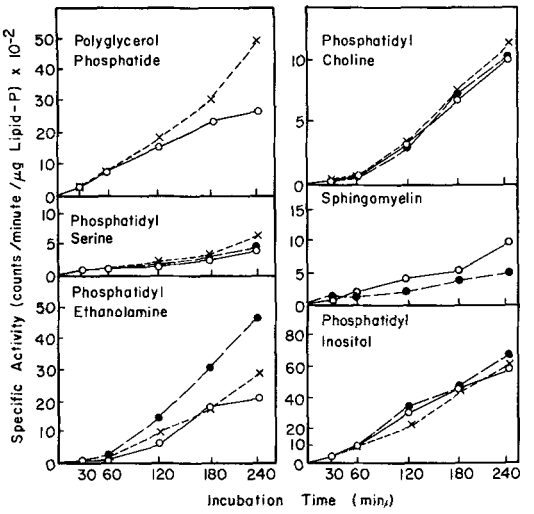

FIG. 5. Specific activity time-curves of individual phospholipids in the subcellular fractions of KB cells. Nine-day old KB cells, grown as monolayer in growth medium containing 10% human serum, were incubated with $^{32}P_i$ (3 μc/ml). Three bottles of cells were pooled for each designated time-interval: (x—x), nuclear; (o—o), mitochondria; and (●—●), microsomal fraction.

The incorporation of $^{32}P_i$ into phosphatidyl choline of mitochondria and microsomes of HeLa and KB cells incubated with the isotope for 3 hrs is similar. Gurr et al. (7) has observed only a small difference in the incorporation of isotope in phosphatidyl choline in mitochondria and microsomes from rat liver three hrs after administration of $^{32}P_i$. However more $^{32}P_i$ is incorporated into the phosphatidyl choline of microsomes of rat liver at six to 12 hrs after administration of the isotope. The greater incorporation of $^{32}P_i$ into phosphatidyl ethanolamine than phosphatidyl choline of microsomes from HeLa and KB cells may suggest that the second pathway of lecithin biosynthesis, involving the methylation of phosphatidyl ethanolamine, is more active. It is apparent from the data of Figs. 4 and 5 that the phospholipids of these subcellular fractions have a high degree of turnover and represent an active process in the membranes of these cellular units and are probably related to the renewal of the biological membrane structures or some other physiological process.

The increased incorporation of the isotope into phosphatidyl inositol and polyglycerol phosphatide in whole cells and subcellular fractions (Figs. 1, 2, 4, and 5) would suggest that these lipids may be involved in some metabolic process of the cell. Hokin and Hokin (10) have suggested that phosphatidic acid and phosphatidyl inositol might serve as a carrier in the active transport of sodium through cell membranes and may be involved in zymogen secretion by the pancreas (9). To substantiate further their role in subcellular fractions Vignais and Lehninger (26) have demonstrated that phosphatidyl inositol is a factor required in mitochondria contraction.

ACKNOWLEDGMENTS

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In Vitro Biosynthesis of Fatty Acids in *Drosophila melanogaster*

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ABSTRACT

A new in vitro technique, utilizing ruptured larvae of *Drosophila melanogaster,* was employed to study the incorporation of ^{3}H-acetate into long-chain fatty acids. Preparative gas-liquid chromatography and scintillation spectroscopy were used to determine the relative activity of each fatty acid from total lipid extracts. Quantitative changes were observed in the distribution of label during the course of the incubation times, which ranged from five minutes to nine hours. All fatty acids which were observed to incorporate acetate in previous in vivo studies also showed incorporation of label under these in vitro conditions. It is concluded that this system may be useful for studying aspects of insect metabolism for short intervals of time.

INTRODUCTION

Drosophilia melanogaster has long been an important organism in the study of genetics; however investigations dealing with its biochemistry have not kept pace. The details and mechanisms of the biosynthesis of fatty acids have been relatively unexplored in *Drosophila* as well as in insects in general.

Keith studied the fatty acid composition of larval *D. melanogaster* lipids and the influence of diet upon this composition, subsequently demonstrating several metabolic interactions under in vivo conditions (1,2). In order to study the mechanisms of some of these biosynthetic reactions it is necessary to employ a system whereby the restrictions of digestion and transport across a gut wall are not limiting. (These limitations can be partially reduced in many organisms by injection of the desired substrate; however injections have limitations in organisms as small as *Drosophila* larvae.)

Literature dealing with the biosynthesis of fatty acids by insects under in vitro conditions is very limited. The fat body of the moth, *Prodenia eridania,* was employed by Zebe and McShan in cell-free preparations (3). Tietz also employed cell-free extracts of the fat body from the migratory locust *Locusta migratoria* (4). In both cases the 16-carbon fatty acids (especially palmitate) were the major components which were formed although 12-, 14-, and 18-carbon fatty acids were also synthesized. Both of the above authors defined the necessary cofactors for cell-free synthesis.

The present investigation employs an in vitro preparation with a minimal amount of tissue injury. This is possible because the fat bodies of *D. melanogaster* larvae, in which the bulk of fatty acid biosynthesis occurs, are only one cell thick at any point in their structure (5). Therefore the fat bodies of ruptured larvae, which are bathed in a liquid medium, offer an excellent opportunity to study fatty acid metabolism, especially for short-term intervals since diffusion into this organ takes place very rapidly.

EXPERIMENTAL

A strain isogenic for Chromosomes I, II, and III of the wild type, Oregon-R of *D. melanogaster,* was employed in all experiments. Eggs were collected from five- to nine-day-old adults onto Petri dishes, filled with a 3% agar-10% sucrose-5% vinegar mixture. Pint jars which contained about 500 adults were inverted on these dishes for two- to four-hour egg-laying periods. These eggs were surface-sterilized and placed onto an axenic medium which was maintained at 25C as previously described (6). Cultures were kept under axenic conditions until the larvae were harvested.

Mid-third instar larvae were washed from the medium, starved two hours, then placed on ice. The body walls were ruptured by employing two jeweler's tweezers in such a manner that the internal organs were exposed and bathed in the liquid culture medium described in Table I. The medium was frozen in one-ml quantities until time for use, and two antibiotics were added to inhibit microbial growth during the course of incubation. One ml of this medium, combined with approximately 0.2 g of ruptured larvae, was placed in a water-bath shaker and equilibrated to 25C. Radioactive substrate was then added. Short-term experiments ($\leq$ 15 min) were stopped with trichloroacetic acid; all others were recovered at the end of the time by filtration and kept frozen until they were extracted.

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Since the incubations were not carried out under sterile conditions, a preparation of ruptured larvae which had been heated to 100C for 15 min was incubated for nine hours and then analyzed in the same fashion. No significant ($<$ 300 cpm in total fatty acid preparations) incorporation was found in the larval tissue fractions or in the supernatant ($<$ 250 cpm).

Lipid extraction was carried out according to the method of Folch et al. (7). Saponification and methylation were performed as described by Böttcher et al. (8). Gas-liquid chromatography (GLC) was carried out on a Perkin-Elmer Model 800 Gas Chromatograph, equipped with a flame ionization detector. Preparative collections of tracer-containing fatty acids were achieved by employing an 80:1 stream splitter distal to the column and proximal to the detector; the smaller split went to the detector and the larger split was collected. Collection tubes were changed manually and were cooled with dry ice during collection. By using collection tubes of different lengths, it was verified that condensation occurred in the first few cms of the tubes even at room temperature. A carrier methyl ester mixture (prepared from larvae) was routinely collected in the same manner and served as a standard background count for each tracer-containing component.

The ^{3}H-Acetate was obtained from Nuclear Chicago. No tracer-containing impurities were found which had a different migration rate from that of acetic acid when thin-layer chromatography (ethanol: NH_4OH solvent on Silica Gel-G) was employed.

TABLE I

Medium for Incubation of Ruptured-Larvae Preparations

Component	g/liter
KCL	7.5
$MgCl_2$	2.5
KH_2PO_4	4.5
Na_2HPO_4	9.0
Nonfat milk solids	25
Na Citrate	8.0
Choline	0.04
Yeast nucleic acid	1.0
Cholesterol	0.2
Thiamine	0.01
Riboflavin	0.01
Nicotinic acid	0.01
Ca Pantothenate	0.002
Pyridoxine	0.002
Biotin	0.0002
Folic acid	0.003
Dihydrostreptomycin	0.06
Penicillin-G	0.2

RESULTS AND DISCUSSION

In previous experiments in this laboratory the distribution of label among various fatty acids was studied in larvae reared on a diet containing ^{3}H-acetate or 1-^{14}C-acetate (2); the distribution was the same for both labels (Table II). All fatty acids which showed incorporation of acetate in those experiments also showed incorporation in the in vitro preparations of ruptured larvae. The percentage distribution of label after nine-hour incubations quite closely resembled the distribution occurring under in vivo conditions (Table II).

The relative amount of tracer in any one component varied considerably with time, and an inspection of Table III shows that saturated and unsaturated fatty acids displayed distinctly different patterns of uptake. Saturated fatty acids showed the highest uptake in the first hour; thereafter the percentage of total activity declined or remained constant. Unsaturated fatty acids were formed to a limited extent in the first hour but increased in percentage of activity throughout the nine hours. (An exception is 14:1, which is a minor component of *Drosophila* lipids.) It has been shown in

TABLE II

Relative Distribution of ^{3}H and ^{14}C from ^{3}H-acetate and 1-^{14}C-acetate into Fatty Acids in vivo[a]

Label	Amount of tracer	Distribution of cpm (%) 12:0	14:0	14:1	16:0	16:1	18:0	18:1
^{3}H-acetate	120 μc to 50 ml food	3.44	23.30	7.29	21.28	22.29	2.13	20.26
1-^{14}C-acetate	40 μc to 50 ml food	3.30	25.31	6.83	21.39	24.23	1.67	17.27
^{3}H-acetate ruptured larvae in vitro	0.1 mc for 9 hr	2.30	19.67	1.21	26.80	17.24	3.68	29.09

[a] The distribution of ^{3}H from ^{3}H-acetate average of seven determinations and ^{14}C from 1-^{14}C-acetate average of four determinations is virtually the same. S. E. of the mean percentage did not exceed 2.3 for any data shown.

TABLE III

Distribution of ^3H from ^3H-acetate into the Fatty Acids of *D. melanogaster*, Employing in vitro Preparations from Five Minutes to Nine Hours[a]

Time in minutes	Amount of ^3H-acetate	Distribution of ^3H cpm (%)						
		12:0	14:0	14:1	16:0	16:1	18:0	18:1
5	1mc	10.58 ±2.3	33.94 ±5.1	3.18 ±1.6	25.57 ±0.82	10.37 ±1.7	7.87 ±1.4	8.51 ±1.4
10[b]	1mc	14.67 ±1.3	30.02 ±6.1	4.81 ±2.57	25.61 ±3.2	8.46 ±1.8	8.65 ±3.1	7.79 ±1.2
20	1mc	12.45 ±0.9	31.39 ±4.3	4.33 ±2.7	24.53 ±1.4	10.76 ±0.6	8.38 ±1.0	8.22 ±0.3
30	1mc	10.99 ±0.4	29.87 ±5.1	1.73 ±0.9	27.55 ±2.2	9.15 ±1.8	8.86 ±1.7	11.78 ±2.8
45	0.5mc	11.06 ±0.4	25.81 ±1.3	3.02 ±2.2	26.31 ±3.7	8.19 ±1.2	10.07 ±0.8	15.53 ±0.2
60	0.2mc	12.34 ±0.7	27.49 ±0.8	3.04 ±1.3	24.53 ±4.7	10.01 ±1.4	7.61 ±1.4	15.33 ±2.6
120	0.2mc	5.71 ±4.0	24.47 ±3.9	5.26 ±2.4	25.63 ±1.0	11.20 ±3.6	9.41 ±0.3	18.32 ±2.9
240	0.1mc	5.79 ±1.2	23.12 ±0.4	1.72 ±0.3	25.52 ±1.6	9.41 ±3.5	7.62 ±2.8	26.33 ±1.4
360[c]	0.1mc	4.02 ±0.6	21.81 ±0.7	2.51 ±1.1	24.69 ±2.4	16.00 ±1.7	5.29 ±1.0	25.69 ±0.4
420[c]	0.1mc	4.54 ±0.9	24.57 ±3.7	2.58 ±1.1	21.42 ±0.5	14.34 ±3.5	5.72 ±1.0	28.33 ±3.2
540[c]	0.1mc	2.30 ±1.1	19.67 ±0.3	1.21 ±0.1	26.80 ±0.7	17.24 ±2.4	3.68 ±0.6	29.09 ±2.2

[a] Mean-percentage distribution of ^3H from ^3H-acetate into fatty acids at the times indicated. The mean is of three determinations except where indicated. Total cpm for the seven components shown was $> 5{,}000$ in all cases except the one which is indicated.

[b] The cpm were 3,144, 14,219, and 18,740.

[c] Mean of two determinations.

previous in vivo work in this laboratory that the monoenes may have as precursors the corresponding saturated fatty acids but that they may also be formed from acetate in an independent manner (2). Thus the curves for 16:1 and 18:1 may represent the sum of two individual pathways as the desaturation of the saturated homolog becomes increasingly important with time. There is strong evidence that the rat (9), many insects other than *Drosophila* (10), and *D. melanogaster* (2) all produce monoenes by more than one pathway.

The steadily rising percentage of activity also is consistent with the previous finding in dietary experiments that monoenes, especially 18:1, once formed, tend not to serve as precursors for other fatty acids. Polyenes are not synthesized by *Drosophila*, nor are fatty acids of longer chain-length than 18 carbons (2).

It is concluded that in vitro preparations of ruptured larvae incorporate acetate by the pathways which feeding experiments show to be operating in the living organism. Hence this system has potential for short-term metabolic studies which are impossible under in vivo conditions.

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Pentane from Thermal Decomposition of Lipoxidase-Derived Products

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ABSTRACT

Thermal decomposition of 13-hydroperoxyoctadeca-9,11-dienoic acid yields hydrocarbons as part of the scission products. Pentane is formed predominately and to the practical exclusion of all other short-chain hydrocarbons.

INTRODUCTION

HYDROCARBONS ARE KNOWN to appear in the early stages of autoxidation of edible fats (1-4). The development of a highly selective and sensitive chromatographic method that allows exclusive determination of hydrocarbons in the presence of volatile polar oxidation products has provided a rapid means of determining the extent of autoxidation (5,6). Hydroperoxides are heat-labile and must undergo some decomposition before derived hydrocarbons are present in the autoxidizing system. Rapid and apparently complete decomposition of the fatty hydroperoxides is achieved by heating the autoxidized mixture to at least 200C. It has been proposed that hydrocarbon formation occurs through splitting of the fatty chain on the alkyl side of the carbon atom containing the hydroperoxide group (1). Other investigators have proposed various splits and free radical rearrangements that yield hydrocarbons among the various autoxidation products (4,7,8).

The exclusive formation of 13-hydroperoxyoctadeca-9,11-dienoic acid by lipoxidase from linoleic acid (9,10) permits compositional studies to be made on the thermal decomposition of a pure hydroperoxide of known structure. Experiments are reported on the formation of hydrocarbons which result from the decomposition of lipoxidase-oxidation products.

EXPERIMENTAL SECTION

The 13-hydroperoxyoctadeca-9,11-dienoic acid was prepared by incubation of lipoxidase with pure linoleic acid as described in previous publications by Dolev et al. (9). The lipoxidase oxidation products were fractionated on a silicic acid column with a methanolic-benzene system, as described by Frankel et al. (11). As determined iodometrically, the peroxide value of the fractionated hydroperoxide, calculated about 8,060 meq. O_2/kg, and UV spectroscopy showed 100% diene conjugation (K_{234} 78.2). The peroxide value reported is above the theoretical value, but, in determining high peroxide values on small samples, large errors are unavoidable.

An autoxidized sample of methyl linoleate (99.8% pure) was used for comparison with the lipoxidized-prepared sample. Autoxidation of pure methyl linoleate (from The Hormel Institute) was conducted at 60C for 27 hr under air with intermittent hand agitation. The peroxide value (iodometric) of this preparation was 362 meq O_2/kg.

Gas chromatographic analyses to determine the hydrocarbon breakdown products of the various fractions were conducted according to methods described by Hoffmann et al. (12) and List et al. (13) with an Al_2O_3 column, which allows passage of the hydrocarbons and irreversibly adsorbs the polar oxygenated materials.

No aliphatic hydrocarbon solvents were used either to extract or fractionate the oxidation products. All solvents were evaporated at low temperatures under a stream of nitrogen. Gas chromatographic control analyses for hydrocarbons were run on all solvents. Diethyl ether was used for extraction; methanol and benzene, for liquid fractionation; and carbon disulfide, for gas chromatographic solutions. Trace amounts of benzene were detected and must be considered in the interpretation of chomatograms.

RESULTS AND DISCUSSION

Fractionation (Fig. 1) of the diethyl ether-soluble lipoxidase-oxidized products from the incubation with linoleic acid showed that 31% of the acid (I) was unreacted and 36% was recovered as pure hydroperoxide (II) plus 25% as a polar-polymeric fraction (IV). An intermediate fraction of 7.3% (III) was discarded. Separation of hydrocarbons by the selective Al_2O_3 gas chromatographic columns is shown

[1]No. Utiliz. Res. Dev. Div., ARS, USDA.

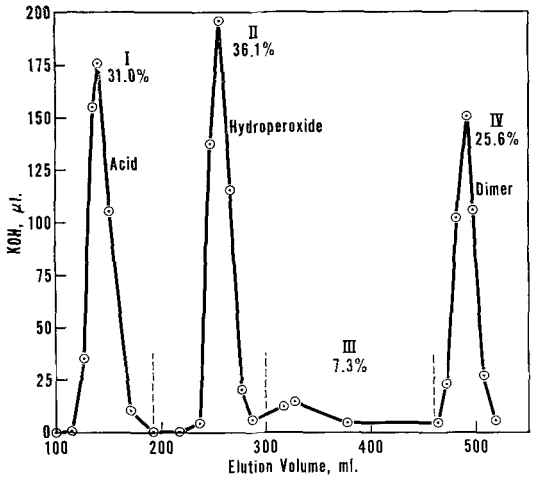


FIG. 1. Separation of lipoxidase-oxidized products of linoleic acid by liquid chromatography on silicic acid. The dimer fraction is assumed to contain small amounts of trimers and higher polymers and polar products.

in Fig. 2A for thermolysis of pure acid hydroperoxide and in Fig. 2B for thermolysis of autoxidized methyl linoleate. To ensure rapid and complete breakdown of hydroperoxides the gas chromatographic injection port, arranged as part of the column extending through the injection heater up to the septum, was operated at 260C. Injecting was done directly into the column.

Pentane is the major volatile hydrocarbon derived from either autoxidized or lipoxidase-derived hydroperoxides. Cleavage of the fatty acid chain on either side of the carbon atom containing the peroxy group results in the formation of a pentane free radical or hexanal

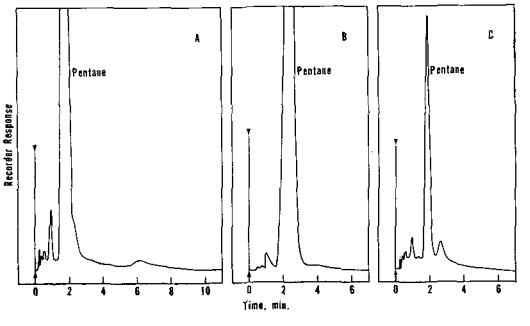
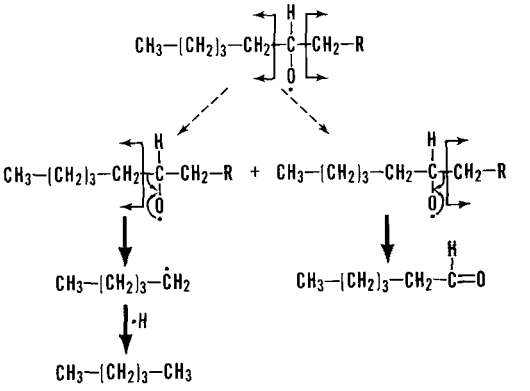


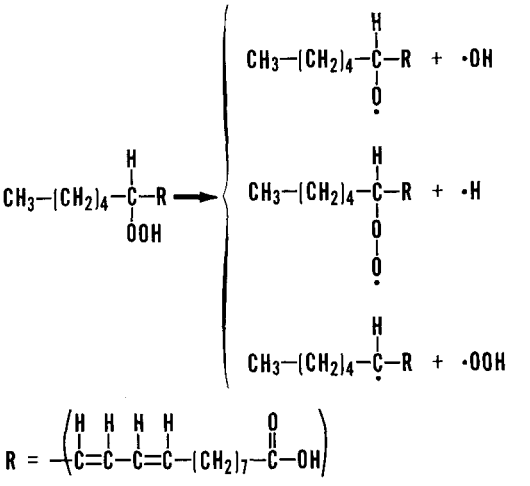
FIG. 2. Hydrocarbons from thermolysis of (A) 13-hydroperoxyoctadeca-9,11-dienoic acid, (B) autoxidized methyl linoleate, and (C) oxidative dimers formed by lipoxidase oxidation of linoleic acid.

(1,2). Hydrogen abstraction by the resulting free radical forms pentane, as shown by the following simplified mechanism:

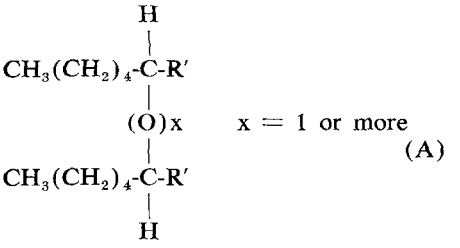


Minor amounts totaling 5% or less of butane, butene, and pentene are indicated in the chromatogram of the lipoxidase-derived product whereas, in the sample from autoxidized linoleate, these components are so small that they are barely indicated. Pentane is also the single major hydrocarbon component derived from the thermal breakdown of the polar-polymeric product which results from lipoxidize oxidation. Fig. 2C, the chromatogram for the polymer, is similar to Fig. 2A for the pure 13-hydroperoxide.

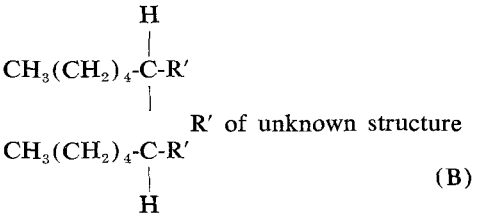
Results indicate that in the oxidative polymer a thermolabile bond exists similar to, and probably derived from, the 13-hydroperoxide linkage. Hydroperoxide decomposition is usually depicted as a free radical breakdown, resulting in formation of one or more of the three following species:



A dimerization between any two of the three species would result in labile products with structure A that would decompose on heating to yield pentane and other products.



A structure such as B would be similar to a thermal dimer,



would be stable to heat, and could be distilled as methyl esters (14). Mixtures of products with these structures would explain the excess oxygen found in the oxidative dimers (11) as well as the fraction of oxidative dimers reportedly similar or identical to thermal dimers (15). The polymer fraction (IV) showed no conjugated diene absorption, indicating that in polymerization the unsaturation had been modified by free radical addition or cyclization. Unequivocal evidence of the structure(s) of oxidative dimers has never been presented, but these postulations are in accord with current theories of peroxide behavior (7,16-19).

The Al_2O_3 gas chromatographic method, highly selective for removal of oxygenated polar materials, is not sufficiently sophisticated because of temperature limitations to determine quantitatively nonane or any of the higher hydrocarbons. Higher-molecular-weight unsaturated hydrocarbons, above C_8 and including monosubstituted acetylenes (above C_5), would not be detected under present operational limits.

Scission of the fatty acid chain between the 13 and 14 carbon atoms of 13-hydroperoxyoctadeca-9,11dienoic acid is well substantiated by these results, which show that pentane is the predominant short-chain hydrocarbon to arise through thermal decomposition. Pentane need not arise from hexanal as a secondary product of autoxidation, as postulated by Buttery et al. (20). Only minimum amounts of pentane are detected unless the oil is heated to temperatures above the decomposition point of the hydroperoxide, and no increase in the amount of the pentane is observed when 0.025% hexanal is added to an oil.

Preliminary work on autoxidized linolenic acid shows that it yields ethane as the major thermolysis product. When derived from hydrogenated fats, the fatty acid hydroperoxides have different-length, saturated, terminal hydrocarbon chains. These hydroperoxides heat-degrade to yield normal hydrocarbons which contain one less carbon atom than was present in the terminal chain of the hydrogenated fatty acid. Hydroperoxides with an unsaturation terminal to the peroxy group should yield unsaturated hydrocarbons. Formation of specific hydrocarbons by thermolysis of oxidized fatty acids and oxidative polymers provides additional information about their composition and structure.

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SHORT COMMUNICATIONS

Thermal Hydrolysis of Some Natural Fats

ALTHOUGH THE ACIDS located in the primary positions of triglyceride molecules are known to be preferentially hydrolyzed by pancreatic lipase, little information is available on whether the release of fatty acids upon thermal hydrolysis is selective with respect to their nature or position. Sahasrabudhe et al. (1) studied the degradation of corn oil heated in air and suggested a greater susceptibility to heat of the primary positions. On the other hand, various other investigators have reported the formation of free acids of approximately the same composition as the parent triglycerides. This communication summarizes the results of a quantitative study of the free fatty acids released in three natural fats upon heating. The fats selected, lard, corn oil, and cottonseed oil, vary in both their fatty acid composition and distribution on the glyceride molecules.

The samples of oil, each approximately 0.5 g to which 10% distilled water was added, were heated at 200C for 4 hr under a nitrogen atmosphere in sealed tubes. The fatty acid composition of each sample was analyzed by the method of Stoffel et al. (2), and the free fatty acids were adsorbed and methylated on a basic resin as described by Hornstein et al (3). Gas chromatographic analysis of the methyl esters was carried out with an F&M Model 609 instrument, equipped with a flame ionization detector and a 15-ft, ⅛-in., diethylene glycol succinate column. Heptadecanoic acid was used as the internal standard. From the analysis of a quantitative mixture of pure fatty acids to which 10% water was added, two correction factors for each acid were obtained, one for the free acid method of Hornstein and the other for the total fatty acid analysis of Stoffel. The percentage of hydrolysis of each acid was calculated from the formula: % hydrolysis $= F_f \times (A\text{-}B)/F_t \times C \times 100$, where

F_f correction factor for free fatty acid analysis
F_t correction factor for total fatty acid analysis
A free fatty acid after heating (mg/g of fat)
B free fatty acid before heating (mg/g of fat)
C total fatty acid initially present (mg/g of fat)

The data were treated by analysis of variance, and the differences among the means were analyzed by the Duncan's multiple range test. The results summarized in Table I indicate a trend towards selectivity of hydrolysis in favor of the shorter chain and the unsaturated acids. In lard the hydrolysis of the $C_{14:0}$ acid was significantly higher than that of all other acids, which showed no significant differences among themselves. In cottonseed oil both myristic and palmitoleic acids were released to a greater extent than the remaining acids, and the linoleic acid in corn oil was hydrolyzed at a significantly higher rate than the other component acids. Although the differences in percentage of hydrolysis among the C_{18} acids were not statistically significant in either lard or cottonseed oil, it is interesting to note that, in all three fats studied, the extent of hydrolysis of the $C_{18:0}$ acid was the lowest and that of the $C_{18:2}$ was the highest.

TABLE I
Hydrolysis of Some Natural Fats Heated at 200C for Four Hours

Lard (17 samples)	% Hydrolysis (means[a])	C_{18} (20.36	$C_{16:1}$ 20.83	C_{16} 20.85	$C_{18:1}$ 20.94	$C_{18:2}$ 21.39)	C_{14} (23.21)
	Specificity of location[b]	$25_{1,3}$	33_2	152_2	$31_{1,3}$	$27_{1,3}$	200_2
Corn oil (22 samples)	% Hydrolysis (means[a])	C_{18} (17.86	C_{16} 18.50	$C_{18:1}$ 18.63)	$C_{18:2}$ (19.62)		
	Specificity of location[b]	$50_{1,3}$	$50_{1,3}$	3_2	27_2		
Cottonseed oil (15 samples)	% Hydrolysis (means[a])	C_{18} (24.42	$C_{18:1}$ 24.46	C_{16} 24.82	$C_{18:2}$ 24.89)	$C_{16:1}$ (27.41	C_{14} 27.84)
	Specificity of location[b]	$50_{1,3}$	24_2	$37_{1,3}$	38_2	....	....

[a] Any two means included in the same bracket do not differ significantly. Any two means not included in the same bracket do differ significantly.

[b] According to Vander Wal et al. (1964). Specificity = % of positive deviation from random distribution. The locations of predominance are shown in subscripts.

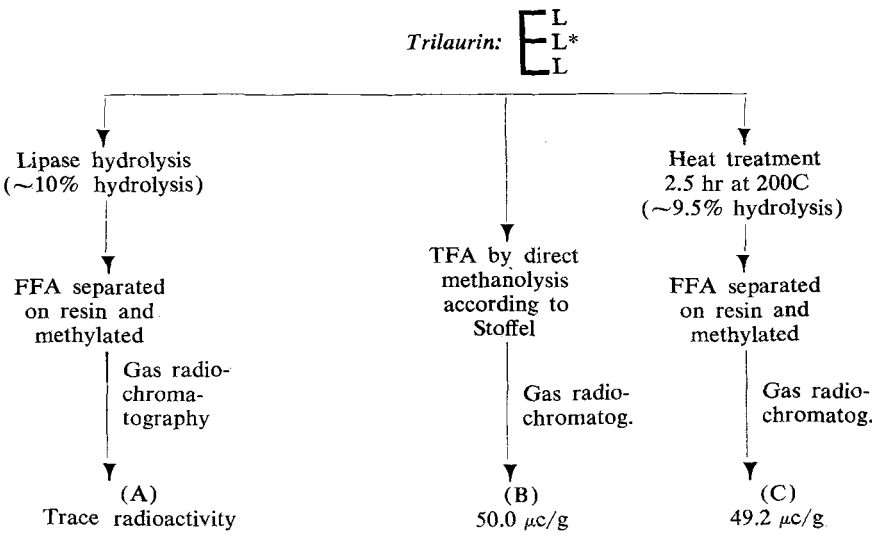

FIG. 1.

No correlation between location of the acid on the glyceride molecule and the extent of hydrolysis can be observed from the data in Table I. For example, the $C_{16:0}$ acid of lard, although like the $C_{14:0}$ acid is highly concentrated in the 2-position, did not exhibit a high rate of hydrolysis.

Experiments in which shorter reaction times were used did not show any preference in the hydrolysis of the α or β acyl groups. In these experiments a trilaurin with the fatty acid in the 2-position labeled with ^{14}C was synthesized, and aliquots were treated according to the scheme below. The methyl esters were analyzed in a combination gas chromatograph-flow counter which permits simultaneous measurement of the acids and their radioactivity. Lipase hydrolysis confirmed that the primary positions were practically free of radioactivity. As shown in Fig. 1, there was no significant difference between the specific activity of the acids released by heat (C) and that of the total fatty acids before heating (B).

Under the experimental conditions used, no randomization or reesterification appeared to occur. This was demonstrated by the use of pancreatic lipase, also by adding free undecanoic acid to trilaurin before heating. After removing the heat-hydrolyzed free fatty acids, no trace of the C_{11} acid was found in the glyceride residue.

The results suggest that the nature of the acid, not its location, is more likely to be involved in thermal hydrolysis. While the data represent the analyses of a relatively large number of samples and demonstrate statistically significant differences in the rate at which certain fatty acids are thermally hydrolyzed, the magnitude of such differences is relatively low. The selectivity of hydrolysis in favor of the shorter chain and the unsaturated acids is probably attributable to their greater water-solubility. It should be pointed out that, in natural fats, a clear-cut relationship between the hydrolysis of an individual acid and such factors as unsaturation, molecular weight, or location on the triglyceride molecule is complicated by the fact that any one variable can be masked or modified by other variables existing in the same system. Further research is being conducted in this laboratory with simple model systems of triglycerides containing specific fatty acids in specific glyceride positions.

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A Simple Method of Preparing Alcohol Acetates, Wax Esters, and Cholesterol Esters

THE EXCESS OF LITHIUM ALUMINUM HYDRIDE, used in the reduction of carboxylic esters to the corresponding alcohols, is decomposed either by water or ethyl acetate (1). Our experience in using ethyl acetate in these reactions indicated partial acetylation of the alcohols, probably because of in-situ formation of lithium aluminum ethylate. This assumption seemed to be confirmed when palmityl alcohol could be quantitatively acetylated with ethyl acetate in the presence of a similar basic catalyst, sodium methylate.

In the present communication the method of preparing alcohol acetates, wax esters, and cholesterol esters in high yields is described.

Five grams of oleyl alcohol were dissolved in 50 ml of ethyl acetate and mixed with 100 mg of sodium methylate. The mixture was agitated vigorously with a magnetic stirrer in a 250-ml flask. The flask was flushed with nitrogen, immersed in a water bath maintained at 70C, and evacuated with a water aspirator at a rate such that the excess of ethyl acetate and ethyl alcohol resulting from the reaction were completely removed in 15 min. The flask was cooled, and the residue was extracted with diethyl ether; the extract was washed free of alkali with water and dried over anhydrous sodium sulfate. The oleyl acetate so obtained was purified by thin-layer chromatography (TLC) (system: silica gel G/toluene) before infrared analysis and a melting-point determination (mp, –17C to –16C).

The reaction between 1.2 g of methyl linolenate and 1.0 g of palmityl alcohol in the presence of 100 mg of sodium methylate (in the absence of any solvent) was carried out as described in the case of oleyl acetate except that the reaction was continued for 1 hr. Palmityl linolenate was purified by TLC before infrared analysis, and the melting point was determined after crystallization from ethanol (mp, 11.5C to 12.5C).

The reaction between 100 mg of cholesterol, purified by the dibromide method, and 70 mg of methyl palmitoleate in the presence of 20 mg of sodium methylate in 15 ml of benzene was conducted in the same manner as de-

TABLE I
Infrared Characterization

Compounds Investigated	Source of Compound	Phase for Spectra	Typical Stretching Vibrations of R—C(=O)—O—R′ C=O	R—C—O	—O—R′
Acetates					
Palmityl acetate	Acetic anhydride-pyridine method	Liquid film	1745 (S) [a]	1242 (S)	1042 (S)
Heptadecanyl acetate	Obtained during reduction of methyl heptadecanoate with $LiAlH_4$ (purified by TLC[b] for spectra)	Liquid film	1745 (S)	1240 (S)	1040 (S)
Oleyl acetate	Present technique	Liquid film	1742 (S)	1240 (S)	1040 (S)
Palmityl acetate	Present technique	Liquid film	1745 (S)	1242 (S)	1040 (S)
Wax Esters					
Palmityl stearate	Acid chloride method	Solid film	1735 (S)	[c]	[c]
Palmityl linolenate	Present technique	Liquid film	1740 (S)	1245 (WM)	1175 (MS)
Palmityl palmitate	Present technique	Solid film	1732 (S)	[c]	[c]
Cholesteryl Esters					
Cholesteryl esters	Blood plasma	Liquid film	1735 (S)	1250 (WM)	1175 (MS)
Cholesteryl palmitoleate	Present technique	Liquid film	1737 (S)	1250 (WM)	1175 (MS)

[a] It is difficult to specify these peaks in solid spectrum. [b] Silica gel G/toluene.
[c] S — strong; WM — weak medium; MS — medium strong.

The spectra were determined with a Perkin-Elmer Model 21 double beam spectrophotometer, equipped with sodium chloride optics.

scribed for palmityl linolenate. Cholesteryl palmitoleate was purified by TLC before infrared analysis. The melting point was determined after recrystallization from acetone (mp, 49.5C to 50.5C).

By the methods described above, the following compounds were also prepared: palmityl acetate, heptadecanyl acetate, palmityl palmitate, and cholesteryl palmitate. The acetates were recovered quantitatively (>98%); the yields of wax esters and cholesteryl esters were 85-90%.

The important infrared absorption peaks characterizing the compounds are given in Table I. The comparison of the mass spectra of palmityl acetate with that of n-docosyl acetate confirmed its identity (2). Both the compounds had the following peaks: M,M-60, M-88, m/e=116, m/e=61, and m/e=43 (base peak attributable to acetyl ion). Both the spectra also showed agreement in other minor peaks.

Alcoholysis for synthesizing alcohol acetates, wax esters, and cholesteryl esters had the following advantages over other procedures: there was a saving of one step in the synthesis of wax and cholesteryl esters by starting with alcohols rather than acetates (3,4); less noxious reagents were used in the preparation of alcohol acetates.

Although a well-known reaction, alcoholysis up to the present time has been used almost entirely with short-chain alcohols, and the present application to high-molecular-weight alcohols, such as wax alcohols and cholesterol, is of considerable value.

ACKNOWLEDGMENTS

The authors are grateful to R. T. Holman and H. Hayes for the assistance rendered in the interpretation and determination of mass spectra, and to J. R. Chipault and Werner Deutsch for interpreting and determining the infrared spectra.

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Extraction of Chromatographically Isolated Fatty Acids from Liquid Scintillation Fluid

THE EXAMINATION of a specific biosynthesized fatty acid is often impeded when a biological system synthesizes a variety of fatty acids and when this quantity is small. It became necessary to develop methods for the isolation and subsequent examination of a particular fatty acid when the amount available was minute. A method was devised by which an individual fatty acid, isotopically labeled, could be recovered from the scintillation fluid after it had been separated as a methyl ester by gas-liquid chromatography and trapped for radio-assay.

The 1-^{14}C-palmitic acid, 16-^{14}C-palmitic acid, and 1-^{14}C-linoleic acid (Nuclear Chicago Corporation) were used to establish the procedure. These were diluted with nonradioactive palmitic or linoleic acid and methylated with diazomethane; separate aliquots ranging from 7 to 70 μg of each acid were passed through a gas-liquid chromatograph. A series 5000 Barber-Colman apparatus was used, employing a glass U tube, 6 ft×6 mm ID, with 15% HIEFF-2BP (ethylene glycol succinate) on Chromosorb W (WA) 80/100 mesh (Applied Science Company) at 185C and nitrogen gas as carrier (flow rate 200 ml/min). A splitter divided the gas effluent so that a portion passed through the flame ionization detector; the fatty acid peak was detected and recorded. Simultaneously the majority of the effluent passed through a Pasteur pipet containing glass wool, moistened with scintillation fluid (5 g 2,5-diphenyloxazole and 0.3g *p*-bis[2-(5-phenyloxazolyl)]-benzene per liter of toluene), which served to trap the fatty acid (Meinertz and Dole, J. Lipid Res. *3*, 140, 1962). Only the peak corresponding to the methyl ester of the radioactive palmitate or linoleate was collected. The contents of the pipet were flushed into a scintillation vial with 12 ml of scintillation fluid and were then radio-assayed on a Packard Tri-Carb scintillation counter. Approximately 70% of the load applied to the column

scribed for palmityl linolenate. Cholesteryl palmitoleate was purified by TLC before infrared analysis. The melting point was determined after recrystallization from acetone (mp, 49.5C to 50.5C).

By the methods described above, the following compounds were also prepared: palmityl acetate, heptadecanyl acetate, palmityl palmitate, and cholesteryl palmitate. The acetates were recovered quantitatively (>98%); the yields of wax esters and cholesteryl esters were 85-90%.

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Extraction of Chromatographically Isolated Fatty Acids from Liquid Scintillation Fluid

THE EXAMINATION of a specific biosynthesized fatty acid is often impeded when a biological system synthesizes a variety of fatty acids and when this quantity is small. It became necessary to develop methods for the isolation and subsequent examination of a particular fatty acid when the amount available was minute. A method was devised by which an individual fatty acid, isotopically labeled, could be recovered from the scintillation fluid after it had been separated as a methyl ester by gas-liquid chromatography and trapped for radio-assay.

The 1-^{14}C-palmitic acid, 16-^{14}C-palmitic acid, and 1-^{14}C-linoleic acid (Nuclear Chicago Corporation) were used to establish the procedure. These were diluted with nonradioactive palmitic or linoleic acid and methylated with diazomethane; separate aliquots ranging from 7 to 70 μg of each acid were passed through a gas-liquid chromatograph. A series 5000 Barber-Colman apparatus was used, employing a glass U tube, 6 ft×6 mm ID, with 15% HIEFF-2BP (ethylene glycol succinate) on Chromosorb W (WA) 80/100 mesh (Applied Science Company) at 185C and nitrogen gas as carrier (flow rate 200 ml/min). A splitter divided the gas effluent so that a portion passed through the flame ionization detector; the fatty acid peak was detected and recorded. Simultaneously the majority of the effluent passed through a Pasteur pipet containing glass wool, moistened with scintillation fluid (5 g 2,5-diphenyloxazole and 0.3g *p*-bis[2-(5-phenyloxazolyl)]-benzene per liter of toluene), which served to trap the fatty acid (Meinertz and Dole, J. Lipid Res. *3*, 140, 1962). Only the peak corresponding to the methyl ester of the radioactive palmitate or linoleate was collected. The contents of the pipet were flushed into a scintillation vial with 12 ml of scintillation fluid and were then radio-assayed on a Packard Tri-Carb scintillation counter. Approximately 70% of the load applied to the column

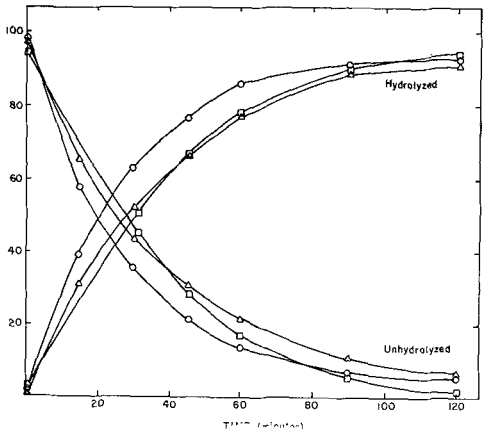

FIG. 1. Percentage of fatty acid radioactivity recovered from the aqueous phase (hydrolyzed) and that present in the scintillation fluid (unhydrolyzed) with time O, 16-^{14}C-palmitic acid; □, 1-^{14}C-palmitic acid; Δ, 1-^{14}C-linoleic acid.

was recovered.

The vial contents were transferred to a 50-ml screw cap tube, and 12 ml of 2.5% KOH in 95% ethanol were added. This one-phase system was heated at 37C for 120 min, 5 ml of distilled water were added, the contents were mixed on a Vortex mixer and centrifuged to separate the two phases.

The top layer (scintillation fluid) was aspirated, and the lower aqueous layer was extracted one time with 10 ml of toluene and three times with 10-ml aliquots of redistilled petroleum ether (bp 40-49C). The extracts containing the scintillation phosphor were discarded, and the aqueous layer was made to pH 1 with concentrated HCl. The aqueous phase was extracted three times with 10-ml aliquots of petroleum ether; the resulting extract accounted for about 94% of the fatty acids initially radio-assayed. When an extract was dried, some contamination by the phosphor was visible but amounted to less than 0.5% of that originally present in the scintillation fluid.

Preliminary testing of this procedure showed that 120 min were sufficient to hydrolyze the methyl esters of the fatty acids. Figure 1 illustrates the rate of hydrolysis, plotting both the percentage of fatty acid methyl ester which remained in the scintillation fluid and that which had been hydrolyzed and subsequently extracted from the lower phase. About 6% of the radioactivity was unaccounted for in the aqueous phase after 120 min of incubation. Incubation beyond 120 min did not significantly increase the yield of fatty acid.

The fatty acids extracted by this method were examined to ascertain if they had been altered. Samples of the fatty acids previously subjected to gas chromatography and recovered were methylated and again passed through the gas-liquid chromatograph. The effluent was monitored to encompass the fatty acid peak, also before each peak appeared and afterward for 20-30 min. In all cases 97% of the radioactivity was associated with the fatty acid peak desired whereas most of the remaining 3% was detected after the peak. Another point of interest was the effect that this extraction procedure might have on fatty acids which were to be decarboxylated (Brady et al., J. Biol. Chem. *235*, 3093, 1960). The percentage of ^{14}C-carbon dioxide recovered from 1-^{14}C-linoleic acid and 1-^{14}C-palmitic acid was the same (<1% difference) before and after the extraction process. About 1% of the ^{14}C from 16-^{14}C-palmitic acid was radio-assayed in the trapped carbon dioxide fraction before gas-liquid chromatography as well as after the extraction procedure.

These results demonstrate that a mixture of fatty acids can be separated and individual ones subsequently examined. The presence of the scintillation phosphor does not affect saponification, extraction, or structure of the fatty acids. This method is being used in current work to examine components of a mixture of biosynthesized fatty acids. Some of these fatty acids are decarboxylated after their recovery from the scintillation fluid. Certain of the unsaturated fatty acids have been hydrogenated (Farquhar et al., Nutr. Rev. Suppl. *17*, 1, 1959) after their extraction, rechromatographed, and re-isolated from the column in the peak corresponding to the saturated fatty acid containing the same number of carbons as the unsaturated fatty acid. Subsequent extraction and decarboxylation yielded amounts of radioactivity within the ranges of theoretical values. The small amount of phosphor extracted does not affect the decarboxylation or hydrogenation procedures.

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Pancreatic Lipase Hydrolysis as a Source of Diglycerides for the Stereospecific Analysis of Triglycerides

BROCKERHOFF (1) AND LANDS et al. (2) have recently published methods for the stereospecific analysis of triglycerides (TG). Since Lands' procedure allows the use of smaller samples, it was selected by the authors. However, when corn oil was analyzed by that method, the results disagreed with the corn oil triglyceride structure reported by Brockerhoff (1). A closer perusal of Lands' published data revealed that his diglyceride (DG) products of lipase hydrolysis contained a higher concentration of saturated acids than would be expected by calculation (Table I) and were thus not representative of the fatty acid distribution in the original TG. Similar divergences have been reported by Luddy et al. (3) and by Coleman (4), but DG data provided by Brockerhoff (1,5,6) (Table I) do not show this enrichment. Possibly some pancreatic lipase hydrolysis techniques may not randomly remove fatty acids from the 1,3-positions of all triglycerides.

In order to test the possible nonrandom removal of fatty acids from the 1- and 3-positions, three different in vitro methods of pancreatic lipase hydrolysis (1–3) were tried on three vegetable oils and on two animal fats containing varied concentrations of saturated, monoenoic, and dienoic acids. When the three methods were applied to the vegetable oils (Table II), only Method B (3) produced DGs of the fatty acid composition expected by calculation. When methods B (3) and C (2) were applied to animal fats, the average data provided by Method B were closer to the calculated values than those provided by Method C, but the individual variations in one case (Rat fat I) were relatively high. Range values for palmitic acid are included in the table as an example.

TABLE I

Deviation from Theory of the Fatty Acid Composition of Diglycerides Obtained by Pancreatic Lipase Hydrolysis of Triglyceride Mixtures

Acid	Original[a] TG	Products of Lipase Hydrolysis[a] MG	DG	(DG) calc.[b]
	mole percentage			
(A)[c]				
14:0	3.1	2.4	2.4	2.9
16:0	25	11	29	22
16:1	6.9	5.7	4.7	6.6
18:0	4.5	—	4.7	3.4
18:1	27	30	26	28
18:2	33	51	33	38
(B)[d]				
14:0	16.6	1.1	11.4	12.5
16:0	4.9	8.0	4.6	5.4
16:1	0.2	0.3	0.4	0.2
18:0	19.1	3.3	16.2	15.2
18:1	10.5	15.4	12.3	11.9
18:2	11.2	16.4	13.1	12.5
18:3	37.5	55.4	42.0	42.4

[a] TG = triglycerides; MG = 2-monoglycerides; DG = 1,-2(2,3)-diglycerides.

[b] (DG)calc. = (3T + M)/4, where (DG)calc. is the theoretical percentage of each particular fatty acid in the diglyceride product of pancreatic lipase hydrolysis, calculated from the fatty acid percentages in the original triglycerides (T) and in the monoglyceride products of lipolysis (M).

[c] Data from (2).

[d] Data from (1).

Stereospecific analysis by Lands' procedure (2) consists of lipase hydrolysis of a TG mixture, stereospecific phosphorylation of one of the DG enantiomers (the 1,2-DG), and subsequent fatty acid analysis of the resulting phosphatidic acid (PA). The distribution of fatty acids among positions 1, 2, and 3 in the original TG can then be determined from the fatty acid compositions of the original TG, the monoglyceride (MG) products of lipase hydrolysis, and the PAs produced by the DG kinase reaction. However, these calculations will be correct only if the fatty acid distribution in the DGs which are used as a substrate in the DG kinase reaction is the same as their distribution in the original TG. If the lipase hydrolysis is selective and the DGs are enriched in saturated acids, the enrichment will be present in the PA made from them, and the calculated amount of saturated acids in the 1-position of the original TG will be greater than that which is actually present. Since the calculations are based on a series of differences, the too-high level of saturated acids observed in position 1 will result in an equally too-low level at position 3.

Pancreatic lipase action on any triglyceride gives a 1:1 mixture of 1,2- and 2,3-DGs whereas the PA which is produced by action of DG kinase on that DG mixture contains the fatty acids from the 1,2-DGs only. The error that results from the use of nonrepresentative DGs may therefore be determined by substituting the DG fatty acid composition for that of PA in Lands' equations for calculating positional distribution in the TG. If the DGs are representative, the calculated composition of the 1- and 3-positions must be the same since the DGs have the average fatty acid composition of the 1,2- and 2,3-positions of the TG.

TABLE II

Degree of Agreement Between Calculated and Lipase Hydrolysis Data for Diglyceride Fatty Acid Compositions

Fat	Acid	Method A (1) DG(det.)	Method A (1) (DG)calc.[a]	Method B (3) DG(det.)	Method B (3) (DG)calc.[c]	Method C (2) DG(det.)	Method C (2) (DG)calc.[a]
				mole percentage			
Corn oil[b]	16:0	12.2	9.1	8.7	8.8	11.3	9.0
	18:0	1.9	1.4	1.4	1.4	2.2	1.5
	18:1	23.3	25.3	24.4	24.7	25.5	24.2
	18:2	62.6	64.2	65.4	65.2	61.1	65.3
Peanut oil	16:0	11.0	8.1	7.9	8.1	11.2	8.2
	18:0	2.8	2.0	2.0	2.0	2.5	2.0
	18:1	47.2	47.7	46.6	46.5	46.7	46.2
	18:2	33.1	38.0	37.4	39.2	35.3	39.4
	20:1	1.5	1.2	1.1	1.2	0.9	1.2
	20:2	1.4	1.1	1.6	1.1	0.8	1.0
	22:1	2.9	1.9	3.3	1.9	1.9	1.9
Safflower oil	16:0	7.2	5.4	5.9	5.3	6.4	5.3
	18:0	2.3	1.6	1.8	1.6	2.0	1.6
	18:1	10.9	10.6	10.8	10.5	10.4	10.4
	18:2	79.5	82.4	81.4	82.7	81.2	82.8
Rat fat I[b]	14:0			1.7	2.3	1.8	2.2
	15:0			0.3	0.6	0.3	0.6
	16:0			25.0[c]	24.1	27.1	23.8
	16:1			21.1	21.4	19.7	21.9
	17:1			tr[d]	tr	tr	tr
	18:0			1.3	1.9	1.8	1.9
	18:1			50.7	49.7	49.3	49.6
	18:2			tr	tr	tr	tr
Rat fat II[b]	14:0			1.4	1.9	1.4	1.9
	15:0			0.1	0.4	tr	0.3
	16:0			26.3[e]	26.5	29.8	26.2
	16:1			11.3	9.8	10.8	10.0
	17:1			0.6	0.5	tr	0.5
	18:0			2.7	2.7	3.5	2.5
	18:1			42.4	42.4	41.3	41.9
	18:2			15.2	15.3	13.1	16.6

[a](DG)calc. = see footnote [b], Table I. [b]Average of duplicate analysis. [c]Range 22.6%-27.4%. [d]Less than 0.1%. [e]Range 25.5%-27.2%.

TABLE III

Effect of the Use of Representative or Nonrepresentative Diglyceride Data on the Calculation of the Stereospecific Distribution of Triglyceride Fatty Acids[a]

Acid	Composition[b] TG (I)	DG (II)	MG (III)	2/3DG (IV)	1/3MG (V)	Positional Distribution[c] 1 (IV-V)	2 (V)	3 (I-IV)
				mole percentage				
(A)[d]								
14:0	3.1	2.4	2.4	1.6	0.8	0.8	0.8	1.5
16:0	25	29	11	19.3	3.7	15.6	3.7	5.7
16:1	6.9	4.7	5.7	3.1	1.9	1.2	1.9	3.8
18:0	4.5	4.7	—	3.1	—	3.1	—	1.4
18:1	27	26	30	17.3	10	7.3	10	9.7
18:2	33	33	51	22	17	5	22	11
(B)[d]								
14:0	16.6	11.4	1.1	7.6	0.4	7.2	0.4	9.0
16:0	4.9	4.6	8.0	3.1	2.7	0.4	2.7	1.8
16:1	0.2	0.4	0.3	0.3	0.1	0.2	0.1	—0.1
18:0	19.1	16.2	3.3	10.8	1.1	9.7	1.1	8.3
18:1	10.5	12.3	15.4	8.2	5.1	3.1	5.1	2.3
18:2	11.2	13.1	16.4	8.7	5.5	3.2	5.5	2.5
18:3	37.5	42.0	55.4	28	18.5	9.5	18.5	9.5

[a]All calculations made on the assumptions stated in the text. Method of calculations from (2).

[b]TG = original triglyceride; DG = 1,2(2,3)-diglyceride; MG = 2-monoglyceride.

[c]Calculated from the composition of the diglyceride products of pancreatic lipase hydrolysis.

[d]A = unidentified natural fat, data from (2).

B = synthetic triglyceride, data from (1).

If the DGs are not representative, the calculated compositions at the 1- and 3-positions will differ and the error in the stereospecific analysis will be at least as great as this difference. It must be kept in mind that this calculation is a check on the random action of pancreatic lipase; it is not a calculation of stereospecificity.

The data in Table III were obtained when the above test was applied to the data in Table I. Although the values for position 1 should be the same as those for position 3 in Sample A, they obviously are not, and the difference is a measure of the selectivity in the pancreatic lipase hydrolysis procedure. It follows then that any calculations based on the PA made from these nonrepresentative DGs will be equally incorrect.

In summary, it is apparent that pancreatic lipase hydrolysis may or may not give DGs useful for reliable stereospecific analysis, depending on yet unknown factors. As a consequence, it becomes necessary to determine the DG composition after each lipase hydrolysis and to compare it with the expected composition. If the two are not the same, it may be concluded that the lipase hydrolysis was selective and not random and the diglycerides cannot be used for stereospecific analysis.

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[Received April 18, 1967]

LETTER TO THE EDITOR

A Note on the Mechanism of Lipoxidase Reaction and the Origin of the Oxygen Incorporated into Linoleate Hydroperoxide

Sir: In the review of prior work for our original manuscript [Dolev, A., et al., Lipids *1*, 293 (1966); Lipids *2*, 33 (1967)], we inadvertently overlooked the paper, "Direct Incorporation of Molecular Oxygen into Organic Material by Respiring Corn Seedlings," by G. J. Fritz, W. G. Miller, R. H. Burris, and L. Anderson [Fritz, G. J., et al., Plant Physiol. *33*, 159 (1958)]. Their report described an experiment with lipoxidase-^{18}O and a linoleic acid (60% purity) which was designed to show "the ability of lipoxidase to incorporate molecular oxygen into the substrate." The observed isotope enrichment, 55% of the theoretical value, "supported the assumption that lipoxidase adds oxygen directly to the substrate."

We wish to acknowledge this prior experiment on lipoxidase which our work has now extended to the use of pure linoleic acid and of $H_2{}^{18}O$, as well as ^{18}O; to the isolation of methyl 13-hydroxystereate after hydrogenation of hydroperoxide; and to the mass spectrometric analysis of the isolated fatty acid methyl esters and of gas-phase oxygen. Our experiments (Dolev, A., et al., op. cit.) demonstrated that "oxygen introduced into the hydroperoxide molecules comes from gaseous phase and not from aqueous phase. Furthermore soybean lipoxidase does not catalyze the exchange between gaseous oxygen and water oxygen."

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[Received March 27, 1967]

If the DGs are not representative, the calculated compositions at the 1- and 3-positions will differ and the error in the stereospecific analysis will be at least as great as this difference. It must be kept in mind that this calculation is a check on the random action of pancreatic lipase; it is not a calculation of stereospecificity.

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In summary, it is apparent that pancreatic lipase hydrolysis may or may not give DGs useful for reliable stereospecific analysis, depending on yet unknown factors. As a consequence, it becomes necessary to determine the DG composition after each lipase hydrolysis and to compare it with the expected composition. If the two are not the same, it may be concluded that the lipase hydrolysis was selective and not random and the diglycerides cannot be used for stereospecific analysis.

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A Comparative Study of the Lipids of Chylomicron Membrane and Fat Core and of the Lymph Serum of Dogs[1]

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ABSTRACT

Thoracic lymph was collected from 13 dogs fed corn oil and butterfat. The chylomicrons were isolated by centrifugation. The lipid composition of the fat core and the membrane of the chylomicron was compared to that of the surrounding lymph serum. The fat cores contained 90-96% triglyceride, 0.7-1.9% free cholesterol, 0.2-0.5% steryl ester, 0.9-3.5% free fatty acid and 1.4-6.1% diglyceride, but no phospholipid. The lipids of the membranes contained 58-75% phospholipid, 20-35% triglyceride, 2-5% free cholesterol, 1-2% free fatty acid, and 2-3% diglyceride, but little or no steryl ester. The membrane phospholipids were made up of 70-90% lecithin, 5-20% phosphatidyl ethanolamine, and 1-3% each of lysolecithin and sphingomyelin. The lymph serum contained 24-47% of total lipid as phospholipid, of which 70-92% was lecithin; the phosphatidyl ethanolamine, lysolecithin and sphingomyelin also present contributed 1-10% each. The neutral lipids of the lymph serum contained 49-75% triglyceride, 2-15% free cholesterol, 6-23% esterified cholesterol, 10-33% free fatty acid and 1-6% diglyceride.

Alterations in dietary fat, or plant sterol supplementation led to lesser changes in the lipids of the chylomicron membranes than in the lipids of any other lymph fraction. The least variation was seen in the phospholipids.

INTRODUCTION

IT HAS BEEN WELL ESTABLISHED that absorbed fat is transported to blood via thoracic lymph in the form of discrete lipid particles, called chylomicrons. On the basis of the known physicochemical properties of the various constituents it has been proposed (1) that the chylomicrons represent microscopic droplets of triglycerides surrounded by a phospholipid-protein membrane. A comparable structure has been advanced (2) for the lipid particles of milk from similar considerations and from actual experimental analyses of the fat cores and membranes of the latter particles. Since there is now good evidence (3) that much of the information in the literature concerning the basic principles of absorption and transport of fat can be related to or interpreted by the mechanism of chylomicron formation, it was deemed necessary to obtain more definite information about the chemical composition of the structural components of the chylomicron and about its overall architecture.

In the initiation of this work, methods previously developed (4) for the study of the structure of the milk fat globules were of great help. Subsequent improvements in the experimental techniques, however, necessitated a reinvestigation of the composition of the milk fat particles (5) before accurate comparisons could be made between the structures of these two natural oil-in-water systems. Eventually inferences could be advanced about possible similarities in the mechanisms of their formation (6). The following report describes the experiments performed to determine the lipid composition of the chylomicrons from corn oil and butterfat chyle. Since the completion of this work essentially identical findings have been published by Zilversmit (7).

MATERIALS AND METHODS

All reagents and solvents were of Fisher Certified Reagent grade and with the exception of the petroleum ether, were used without purification. Petroleum ether (bp 30-60C) was redistilled and the fraction boiling in the range 40-56C was collected and used.

Female mongrel dogs, 3 to 5 years old, were purchased from the Canadian Breeding Laboratories, St. Constant, Quebec. The thoracic ducts were cannulated by the method of Beschel and McCarthy (8) under Nembutal anesthesia. Table I gives the details of the experimental meals and lymph collections. The lymph was processed immediately after collection. Occasionally, dilute solutions of heparin were injected in the thoracic duct cannula in order to dislodge clots.

[1] Taken in part from a PhD Thesis submitted by T. C. Huang to Queen's University, Kingston, Canada, in April 1965. Presented at the AOCS 56th Spring Meeting, Houston, May 1965.

TABLE I

Experimental Meals and Lymph Collections[a]

Dog	Meal	Hours of collection[b]	Volume of lymph (ml)	Chylomicron lipid (g)
	Corn Oil Feeding			
A	50 g corn oil	5 — 9	90	0.78
B	50 g corn oil	8 — 17	85	5.07
C	50 g corn oil	3 — 10	85	1.93
D	60 g corn oil	3 — 12	120	2.54
E	60 g corn oil + 3 g plant sterol[c]	4 — 15	270	9.58
F	60 g corn oil + 3 g plant sterol[c]	5 — 19	250	3.58
	Butterfat Feeding			
G	80 g butter	4 — 10	160	6.33
H	70 g butter	6 — 27	255	13.89
I	70 g butter	4 — 8	92	2.0
J	70 g butter	5 — 12	184	4.69
K	55 g butter	11 — 18	90	4.79
L	60 g butter	15 — 18	60	4.66
M	70 g butter + 3 g plant sterol[c]	3 — 9	270	8.65

[a] In addition to the fats all dogs received 2 slices each of white bread.

[b] Hours after feeding the meals.

[c] A mixture of 40% campesterol and 60% β-sitosterol.

Fractionation of Lymph

The method adopted for work with the lymph chylomicrons was based upon that described by Cole et al. (4) for the preparation of the membranes and fat cores of milk fat globules. Fresh lymph was divided into chylomicrons and lymph serum by centrifugation at 36,000 g for 1-2 hr at 5C. A hole was made through the solid layer of chylomicrons at the top of the tube with the aid of a small probe, and a fine-tip glass capillary, connected to a suction pump, was carefully introduced into the liquid lower layer and the serum siphoned off. Recentrifugation of the lymph serum as before showed that it was essentially free of chylomicrons. The chylomicrons were suspended in 0.9% NaCl and recentrifuged. Washing with saline was repeated once more and the compact chylomicron layer was frozen at –4C and left at this temperature for periods of 24-48 hr. The frozen fat particles were then allowed to thaw and the fat melted at 37C was dispersed in 2 volumes of distilled water of the same temperature. The suspension was centrifuged at 36,000 g for 2 hr. The membranous material sedimented to the bottom of the tube as a pellet, while the released oil floated to the top of the tube. The oil phase was collected with the aid of a Pasteur pipet and washed several times with saline to remove adhering unbroken chylomicrons. The membrane pellet was dispersed in distilled water and the resulting suspension again centrifuged. At the centrifugal force of 36,000 g, only a portion of the chylomicron membranes was sedimented in 2 hr. Part of this material remained floating and was collected separately. Subsequent chemical analyses showed that both sedimented and floating membranes had the same lipid composition.

Methods of Lipid Analysis

The lipids of the lymph serum and the chylomicron fractions were extracted either by chloroform-methanol (2:1, v/v) or ethanol-diethyl ether (3:1, v/v) as previously described (9). The denatured and completely precipitated protein was removed by centrifugation, dried with diethyl ether in vacuo, and weighed. The lipid extracts were evaporated to dryness, taken up in chloroform and separated into neutral and phospholipids by chromatography on silicic acid with chloroform and methanol. Further separations were made by TLC (10). Neutral lipids were resolved into individual chemical classes by TLC on silica gel G using heptane-isopropyl ether-glacial acetic acid (60:40:2, v/v/v) as the developing solvent. Phospholipids were resolved into individual chemical classes by TLC on silica gel G using chloroform-methanol-glacial acetic acid-water (65:25:8:4 v/v/v/v) as the developing solvent. The bands were located by spraying the plate with 0.05% 2,7-dichlorofluorescein in methanol and viewing it under ultraviolet light. The various lipid fractions were methylated in the presence of the silica gel scrapings using 10% (w/v) sulfuric acid in methanol (10). To prevent possible decomposition of sterols in the acid trans-

TABLE II

Distribution of Total Lymph Lipid After Destabilization of Chylomicrons[a]

Dog	Total lipid, g	Fat core, g	Globule membrane, g	Globule membrane, %[b]	Lymph serum, g	Unbroken chylomicrons, g	Unbroken chylomicrons, %[c]
			Corn oil feeding				
A	0.86	0.51	0.08	13.8	0.08	0.19	20.0
B	5.94	4.33	0.30	6.4	0.87	0.44	7.3
C	2.31	1.31	0.09	6.2	0.38	0.54	23.3
D	3.12	0.79	0.09	11.3	0.58	1.75	56.0
E	10.96	6.55	0.24	3.6	1.39	2.78	25.2
F	4.56	1.92	0.11	5.5	0.98	1.55	34.1
			Butterfat feeding				
G	7.17	4.19	0.37	8.1	0.84	1.77	24.7
H	15.87	8.37	1.21	14.0	1.99	4.31	27.0
I	2.54	0.96	0.04	3.7	0.47	1.08	42.2
J	5.57	2.75	0.43	13.4	0.88	1.51	27.1
K	5.79	2.38	0.25	9.6	1.00	2.16	37.6
L	5.09	1.86	0.15	7.6	0.43	2.64	52.5
M	10.10	4.79	0.52	9.8	1.45	3.34	32.3

[a] Weights of total lipid extracts.

[b] Percentage of total lipid in the broken chylomicrons.

[c] Percentage of the total chylomicron weight.

methylation medium, the fatty acid moiety of the steryl esters was transmethylated with 0.5 N KOH in anhydrous methanol at room temperature (12 hr).

Gas chromatography of fatty acid methyl esters was performed on an Aerograph Hy-Fi Model B instrument. An F & M Model 40 linear temperature programmer was used to maintain an isothermal temperature of 185C. The injector temperature was 250C. Separations were achieved on a 1/8 in. O.D. X 6 ft stainless steel column packed with 80-100 mesh Gas-Chrom A coated with 20% (w/w) diethylene glycol succinate. Nitrogen supplied at 12 psi gave a flow rate of 80 ml/min at room temperature. Under these conditions, quantitative results with National Heart Institute Fatty Acid Standards (A to F) agreed with the stated composition data with a relative error less than $\pm 2\%$ for major components (more than 10% of total mixture) and less than $\pm$ 10% for minor components (less than 10% of total mixture). For quantitative estimation of fatty acids in the individual lipid classes, a known amount of methyl heptadecanoate (Applied Science Laboratories, Inc., State College, Pa.) was added to each fraction, and the weight of the unknown was deduced from the proportions of the peak areas in comparison to the area of the internal standard. The original weights of the individual lipid classes were calculated from the fatty acid data and the known molecular weights of the nonfatty acid moieties of these molecules (11). Sterols were quantitatively estimated by gas chromatography on a 1/8 in. O. D. × 2 ft stainless steel column packed with 3.8% (w/w) SE-30 on Diatoport S (80-100 mesh) using cholestane as internal standard. For these separations the above gas chromatograph was operated at 220C with an injector temperature of 250C. In addition GLC of intact steryl esters and triglycerides was performed on a Beckman GC-4 gas chromatograph equipped with a special injector heater and dual 3% JXR columns (12).

RESULTS AND DISCUSSION

Separation of Chylomicron Membranes and Fat Cores

In contrast to the large milk fat globules analyzed by Cole et al. (4) the smaller chylomicrons could be destabilized only with difficulty. The extent of destabilization seemed to depend upon such factors as the length of time taken for freezing, the duration of thawing, the water content of the chylomicron cream, and the number of cycles of freezing and thawing employed. The degradation achieved in the 3 cycles usually employed varied from 48-93%. Table II gives the distribution of total lymph lipids among the chylomicron membranes, fat cores, and lymph sera, as well as that retained in the unbroken portion of chylomicrons. A total of 13 samples of lymph, 6 from corn oil and 7 from butter feeding, were examined. As judged from the proportion of the unbroken chylomicrons, the corn oil chylomicrons were somewhat more easily destabilized than the butterfat chylo-

TABLE III

Lipid Composition of Chylomicrons and Lymph Serum
(% total lipid of fraction)

Dog	Fat core		Membrane		Serum	
	PL[a]	NL[b]	PL[a]	NL[b]	PL[a]	NL[b]
			Corn oil feeding			
A	0	100	61.0	39.0	44.5	55.5
B	0	100	58.1	41.9	28.5	71.5
C	0	100	72.3	27.7	31.4	68.6
D	0	100	64.1	35.9	24.8	75.2
E	0	100	75.6	24.4	26.1	73.9
F	0	100	69.0	31.0	25.3	74.7
			Butterfat feeding			
G	0	100	65.3	34.7	47.0	53.0
H	0	100	65.9	34.1	27.1	72.9
I	0	100	68.6	31.4	38.0	62.0
J	0	100	61.9	38.1	30.4	69.6
K	0	100	62.8	37.2	33.0	67.0
L	0	100	60.3	39.7	33.2	66.8
M	0	100	72.9	27.1	33.3	66.7

[a] Phospholipids.

[b] Neutral lipids plus free fatty acids.

microns. Furthermore, it frequently appeared as if additional cycles of freezing would produce no significant increase in the amount of material destabilized. Despite considerable effort expended in comparative studies between the lipids of the lysed and unlysed chylomicrons, no significant differences could be uncovered. If real differences in the stability of these chylomicrons existed, they must have been related to possible discrepancies in their size and/or the amount and composition of the associated proteins. Zilversmit (7) has also reported that at certain temperatures corn oil chylomicrons are more easily oiled out than butterfat chylomicrons, but that heating to 40-60C or higher produces comparable oiling out of both types of chylomicrons.

Table III lists the relative proportions of phospholipids and other lipids in the membranes and fat cores of the chylomicrons and in the lymph sera. In both corn oil and butterfat chylomicrons, all the phospholipids occurred in the membranes and none in the fat cores. Furthermore, the amount of the total phospholipid in the membranes appeared to be about the same in both types of chylomicrons and accounted for 60-70% of the total lipid. There also was little difference between the phospholipid proportions of the lymph sera obtained from corn oil and butterfat feeding. The average percentage of total phospholipid in the lymph sera was 30. This is rather low when compared to fasting lymph which contains about 50% phospholipid, and suggests that considerable amounts of the smaller chylomicrons remained in the lymph serum following centrifugation. In 2 lymph samples from corn oil feeding (A and G), however, the phospholipid content was 44.5 and 47.0% of the total lipid.

Phospholipids

Table IV gives the composition of the phos-

TABLE IV

Composition of Phospholipids of Chylomicron Membrane and Lymph Serum

Dog	Lymph fraction	% total phospholipid			
		Cephalin[a]	Lecithin	Lysolecithin	Sphingomelin
		Corn oil feeding			
A	Membrane	4.0	90.0	2.5	3.5
	Serum	4.9	84.5	6.8	3.8
B	Membrane	3.8	92.0	4.0	0.2
	Serum	8.2	85.0	3.2	3.6
C	Membrane	17.9	79.2	1.2	1.7
	Serum	2.6	86.3	9.6	1.5
D	Membrane	6.4	88.6	1.8	3.2
	Serum	4.3	87.6	3.4	4.7
E	Membrane	23.6	70.0	3.1	3.3
	Serum	3.3	86.5	5.7	4.5
F	Membrane	12.8	83.4	1.2	2.6
	Serum	2.7	80.0	9.8	7.5
		Butterfat feeding			
G	Membrane	13.0	79.1	4.3	3.6
	Serum	4.4	90.5	2.6	2.5
H	Membrane	15.0	79.1	3.2	2.7
	Serum	5.9	78.0	10.1	6.0
I	Membrane	9.4	83.9	1.4	5.3
	Serum	2.6	92.0	3.5	1.9
J	Membrane	4.6	90.5	3.6	1.3
	Serum	2.2	88.1	5.3	4.4
K	Membrane	8.4	79.3	9.4	2.9
	Serum	3.8	88.1	3.7	4.4
L	Membrane	13.8	82.5	2.0	1.7
	Serum	4.3	87.3	6.1	2.3
M	Membrane	8.1	81.2	4.1	6.6
	Serum	4.8	85.8	4.1	5.3

[a] Phosphatidyl ethanolamine plus any phosphatidyl serine.

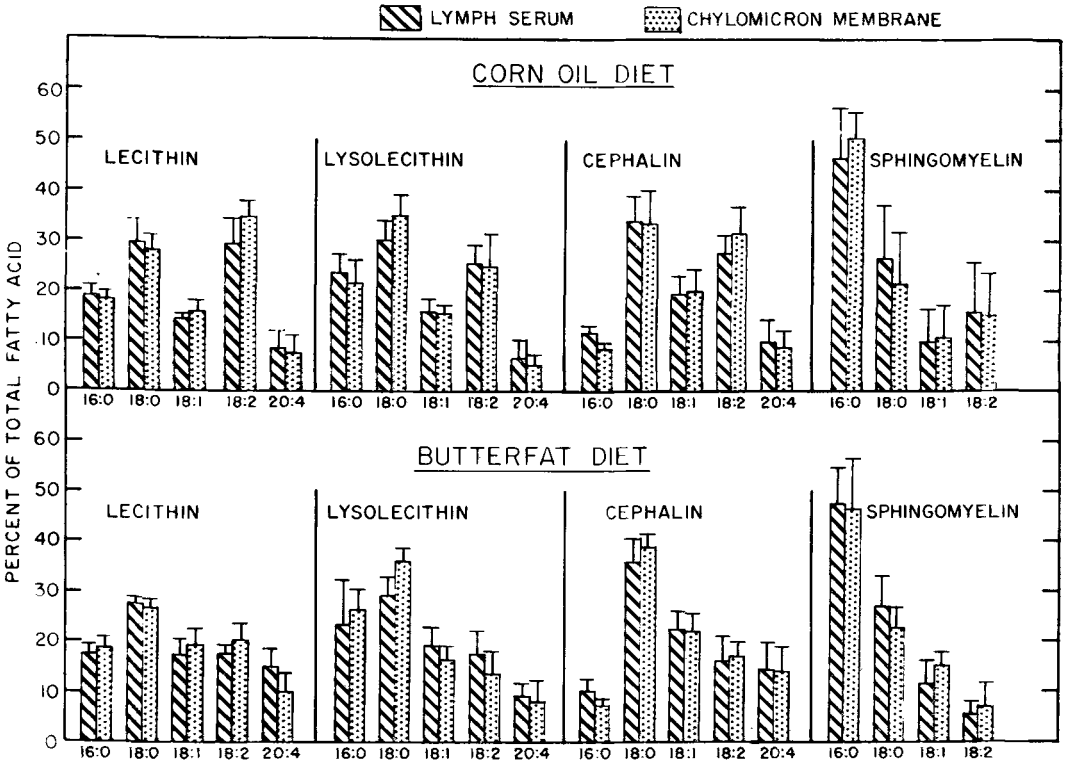

FIG. 1. Fatty acids of individual phospholipids from lymph serum and chylomicron membrane (wt %). Cephalin represents phosphatidyl ethanolamine plus any phosphatidyl serine. Fatty acids identified by the total number of carbons: total number of double bonds. T lines indicate maximum, bars minimum value for the dogs in each group.

pholipids of chylomicron membranes and lymph sera. Since the TLC system employed did not permit a reliable resolution of phosphatidyl ethanolamine and phosphatidyl serine, a combined estimate of the two has been entered under the name of cephalin. Zilversmit (7), however, has shown that it is mainly phosphatidyl ethanolamine. The major phospholipid of the chylomicron membrane was lecithin. It accounted for an average of 85% of the total phospholipid. The cephalins made up an average of 10% of the total phospholipid, although individual estimates ranged from 4-23%. Lysolecithin and sphingomyelin made significantly smaller contributions to the total membrane phospholipids accounting for an average of 3% each. All of these values are remarkably close to those found by Zilversmit (7), who isolated phosphatidyl choline (75%), phosphatidyl ethanolamine (12%), sphingomyelin (5%) and lysolecithin (3%) from similar membrane preparations. Neither Zilversmit nor we could demonstrate any significant differences between the phospholipid compositions of the membranes of corn oil and butterfat chylomicrons.

The proportions of the membrane phospholipids were closely paralleled by the proportions of the lymph serum phospholipids. In most samples, however, the proportion of the cephalins found in the lymph serum was only about one half of that found in the membrane. There were other samples where these proportions were reversed. The changes in the contributions of the cephalins were reflected in slight alterations in the lecithin concentration; the amounts of the lysolecithins and sphingomyelins remaining about the same or increasing slightly over those in the membrane phospholipids. The values for the lymph serum phospholipids tend to approach those for the dog blood serum which is known (13) to contain relatively less phosphatidyl ethanolamine and more sphingomyelin and lysolecithin.

Fig. 1 shows the results of GLC analyses of the fatty acids of individual phospholipid fractions from the chylomicron membranes and

the lymph sera. This bar graph does not include various minor fatty acids also present in these samples which accounted for 5-10% of the total acid mixture. In addition to palmitoleic acid, small amounts of C_{10}-C_{14} saturated and some C_{20}-C_{24} saturated and unsaturated acids were also excluded. Occasionally the neglected longer chain acids alone made up about 10% of the total fatty acids of sphingomyelin.

In general the fatty acid composition of the phospholipids of both lymph serum and the chylomicron membrane remained relatively unaffected by changes in dietary fat. There was, however, a significantly greater proportion of linoleic acid in the phospholipids from the corn oil lymph than in those from the butter lymph. Furthermore this difference was about equally pronounced in the phospholipids of the membranes and the lymph sera. The lecithins and cephalins from both sources contained about equal proportions of saturated and unsaturated fatty acids, while the sphingomyelins contained primarily saturated fatty acids. The composition of the sphingomyelin acids is unusual in the relative absence of the C_{20}-C_{24} acids, which are known to make up 20% or more of the total acids of this fraction from dog blood serum and fasting human plasma. Similar observations regarding the fatty acid composition of these phospholipids were reported by Zilversmit, who examined considerably fewer samples. In contrast to the results of Zilversmit, our samples of lysolecithin contained significantly more of the unsaturated fatty acids amounting to nearly 50% of the total. This observation is unexpected; they should have contained mostly saturated fatty acids, if the present concepts of the composition of the natural phosphatidyl glycerides are correct (14). In view of the effective thin-layer separations, it is suggested that the unsaturated lysolecithins in our samples originated from a nonspecific hydrolysis of the large amounts of the lecithin.

Neutral Lipids and Free Fatty Acids

Table V gives the composition of the neutral lipids and free fatty acids of the fat cores and membranes of the chylomicron and the lymph serum. Triglycerides comprised the bulk (87-96%) of the fat core. Of the remainder, 0.7-1.9% was free sterol, 0.1-0.4% steryl ester, 0.9-5% free fatty acids and 2-6% diglycerides. The relatively constant proportion of free sterol in the fat cores is interesting in view of the difference in the sterol content of the dietary fats. The present value, however, is 2-3 times higher than that reported by Zilversmit (7), who used the $FeCl_3$-H_2SO_4 reagent in his sterol determinations. Apparently one is dealing with relatively small quantities of material which can be measured only with great diffculty in the presence of large amounts of other lipids.

The relatively small fraction of the steryl ester in the fat core is interesting in view of its fatty acid composition to be discussed later. The occurrence of small amounts of free fatty acids and diglycerides in the chylomicron lipid has been reported before (3), although their distribution between the fat core and the chylomicron membrane had not been established. It should be noted that neither free fatty acids nor diglycerides were found in the chylomicron

TABLE V

Composition of Neutral Lipids of Chylomicrons and Lymph Serum[a] (% total neutral lipid)

	Steryl esters			Free sterols			Triglycerides			Free fatty acids			Diglycerides		
Dog	C	M	S	C	M	S	C	M	S	C	M	S	C	M	S
							Corn oil feeding								
A	0.2	tr	19.6	1.2	5.2	8.6	94.6	87.1	48.5	1.7	4.4	16.5	2.3	3.3	6.8
B	0.4	tr	5.6	1.2	4.9	2.1	96.1	86.6	72.1	0.9	4.6	15.5	1.4	3.9	4.7
C	0.2	tr	9.8	0.7	5.9	4.0	96.0	85.2	61.0	1.3	5.1	19.1	1.8	3.8	6.1
D	0.3	tr	5.8	1.1	5.2	2.5	91.0	86.3	57.5	3.5	5.5	33.1	4.1	3.0	1.1
E	0.3	tr	8.3	1.5	8.9	3.1	94.9	79.0	67 8	1.1	7.8	17.4	2.2	4.3	3.4
F	0.3	tr	12.4	1.3	12.1	5.7	93.2	81.9	53.4	1.7	4.2	17.6	3.5	1.8	10.9
							Butterfat feeding								
G	0.3	tr	15.1	1.1	3.9	4.4	95.3	82.5	74.6	1.3	9.5	4.4	1.8	4.1	1.5
H	0.3	tr	10.5	1.1	5.9	5.0	90.2	78.0	75.0	3.2	10.1	7.2	5.2	6.1	2.3
I	0.4	tr	11.2	1.4	4.7	5.5	91.2	81.3	69.3	2.0	6.8	11.5	5.0	7.2	2.4
J	0.5	tr	13.5	1.9	8.1	7.9	90.3	80.0	58.5	1.7	6.6	16.0	5.6	5.3	4.1
K	0.4	tr	14.9	1.3	5.5	2.7	90.2	87.1	54.5	3.0	4.4	25.8	5.1	3.0	2.1
L	0.3	tr	10.4	1.5	9.8	3.5	87.1	76.4	73.0	5.0	7.8	11.0	6.1	6.0	2.1
M	0.2	tr	23.1	1.7	6.2	14.5	91.1	88.1	49.3	1.9	3.2	9.7	5.1	2.5	3.4

[a] C, fat core, and M, membrane of chylomicron. S, lymph serum.

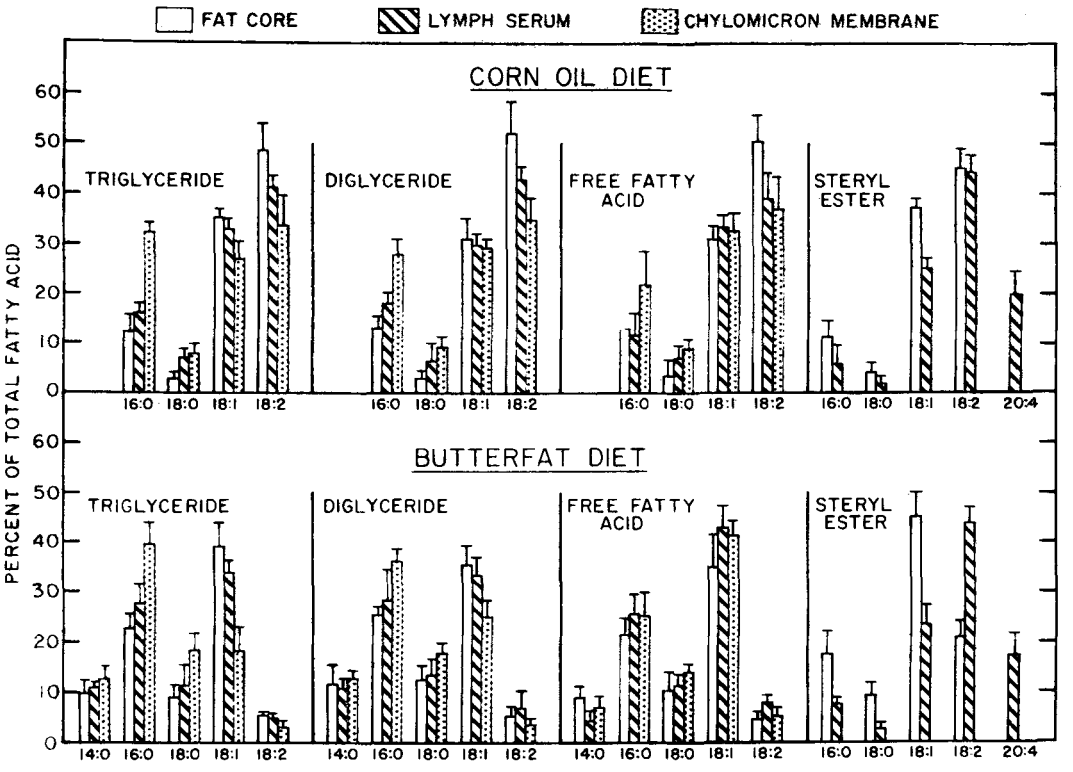

FIG. 2. Fatty acids of neutral lipids of chylomicron fat core and membrane, and of the lymph serum. Legends as in Fig. 1.

lipids examined by Zilversmit (7).

Of the nonphospholipids of the chylomicron membranes, the triglycerides were the major components (76-88%); free fatty acids (4-10%) and diglycerides (3-7%) occurred in about the same proportions in relation to the triglycerides as they did in the fat cores; while the proportion of free sterol was considerably higher (3.9-12.1%). There was little or no steryl ester in the chylomicron membrane. Apparently the free sterol became associated with either the phospholipids or the membrane proteins; whereas the steryl esters did not. Both relative bulkiness and a lack of polarity might have excluded the steryl esters from a tightly organized membrane structure. Assuming that about 10% of the total chylomicron lipid was present in the membrane, it may be calculated that the sterol associated with the membrane accounted for about 50% of the total chylomicron sterol. This estimate is somewhat lower than that of Zilversmit (7), who found that 64-77.8% of the total chylomicron sterol was in the membrane. Such estimates of the distribution of the sterol between the membrane and the fat cores, however, are liable to vary considerably with the size of the fat particles as well as with the availability of sterol during the formation of the membranes. The presence of plant sterols in some of the diets led to the incorporation of relatively small amounts of these compounds into the chylomicron membranes (5-10%).

The nonphospholipid fraction of lymph serum was very different from those of the fat core and the membrane of the chylomicron. An unusual feature of the lipids of these lymph sera was the high proportion of free fatty acids (4-33% of total neutral lipid).

Although no comprehensive studies of the origin and nature of the free fatty acids in the lymph have been carried out, it would appear that in most samples the values (10-15%) are only about twice those (5%) for the free fatty acids of plasma when expressed as per cent of the total nonphosphatide lipid. The erratic increases could have been due to variable experimental stresses, or to an occasional activation of the lipases in the lymph by the infusion of small amounts of heparin

solution. In the latter case the free fatty acids and the plasma triglycerides, as expected, had common fatty acid patterns as shown below. However, small amounts of the dietary fatty acids also could have entered the lymph in the free form (15).

The triglycerides made up 50-75% of the nonphosphatide lipid of the lymph serum, while the steryl esters accounted for an average of 10-20%. On the average, the feeding of butterfat resulted in a larger proportion of cholesteryl ester in the lymph serum lipids. The changes in dietary fat appeared to affect least the proportion of the free sterol in the lymph serum which varied from 3-9%, except for one value of 14.5%. Apparently the somewhat larger amounts of cholesterol ingested and absorbed with butterfat were incorporated mainly in the steryl ester fraction of the lymph serum.

Fig. 2 shows the results of the GLC analyses of the fatty acids of the neutral lipids and free fatty acids of the chylomicron fat cores and membranes, and of the lymph serum. In general the fatty acid composition of these lymph lipids reflected the composition of the dietary triglycerides. Thus the various lipid classes from corn oil lymph were richer in linoleic acid and those from butterfat lymph contained greatly increased proportions of myristic acid. Furthermore, the triglyceride, diglyceride, and free fatty acid classes contained the acids in nearly the same proportions as in the diet, but there were exceptions. The triglyceride fraction of the chylomicron membrane was considerably more saturated than that of the fat core or the lymph serum. The membrane triglycerides were specifically characterized by increased proportions of palmitic acid. A dietary influence, however, was evident in the presence of significant amounts of myristic or linoleic acids in membrane glycerides from butter or corn oil feeding, respectively. As a result of the enrichment in the palmitic acid content, the membrane triglycerides were of a lower average molecular weight than those of the lymph serum or the fat core of the corresponding diet. Because of the higher saturation of the lymph lipids from butter feeding, the difference between the triglycerides of the membranes and fat cores of these chylomicrons was less pronounced than that for the corn oil chylomicrons. Nevertheless, notable differences in the composition of the two triglyceride classes could be demonstrated by the GLC of intact triglycerides. These observations are in agreement with the finding of predominantly saturated glycerides in the membranes of dog, human and rat chylomicrons by Zilversmit (7) and with the isolation of high melting point triglycerides from the membranes of the milk fat globules (16). Whether these observations represent a preferential accumulation of saturated triglycerides in the membrane phase during freezing and thawing, or are related in some manner to critical spatial requirements in the tightly packed membrane, cannot be immediately answered. Attempts to demonstrate similarities between the high melting point membrane triglycerides of corn oil and butterfat chylomicrons by further purification of these glycerides and a GLC examination have thus far proved unsuccessful.

The most dramatic differences in the fatty acid compositions were noted for the steryl esters of the different lymph fractions. In general the steryl esters of the fat cores contained about the same fatty acids as the surrounding triglycerides. The steryl esters of the lymph serum had a more constant fatty acid composition. At no time, for example, were any of the steryl esters of the fat cores seen to contain arachidonic acid, which accounted for about 20% of the total acids of the steryl esters of the lymph sera. Furthermore, the dietary fat had very little influence upon the fatty acid composition of the steryl esters of the lymph serum. An exception was provided by myristic acid, which was occasionally found to be incorporated into the serum steryl esters in amounts of up to 10% of the total. This observation, of a heterogeneity in the lymph steryl esters has not been previously made and requires further study. Fatty acid analyses of lymph steryl esters made by Zilversmit (7) gave widely divergent values and were not further commented upon.

Relation of Protein to Lipid in Chylomicron Membrane

Table VI compares the proportions of total lipid and protein in the chylomicron membranes. Possibly because of the single wash during the preparation, the contribution of the protein is quite variable, although in most samples it accounts for 20-30% of the total weight of the chylomicron membrane. Many of the values, however, are very close and suggest some sort of constancy. Most of them are about 2-3 times higher than the highest value (11.8%) reported by Zilversmit (7), who washed his chylomicrons two times or more. This ratio between the values from the two laboratories is maintained when the protein proportion is related to the total weight of the chylomicron. Since the average weight of the chylomicron

TABLE VI

Relation of Protein to Lipid Content of Chylomicron Membrane

Dog	Total weight of membrane (mg)	Weight of component		Protein/lipid
		Protein %	Lipid %	
		Corn oil feeding		
A	92.9	12.1	87.9	0.14
B	427.9	31.0	69.0	0.45
C	109.1	21.2	78.8	0.27
D	13.0	30.8	69.2	0.45
E	318.4	23.6	76.4	0.31
F	156.1	28.4	71.6	0.40
		Butterfat feeding		
G	528.0	29.9	70.1	0.43
H	1523.2	20.5	79.5	0.26
I	48.1	23.3	76.7	0.30
J	589.6	31.0	69.0	0.45
K	352.0	29.6	70.4	0.42
L	171.4	14.0	86.0	0.16
M	718.5	27.8	72.2	0.39

membrane varies from 5-10% of the total chylomicron weight (as calculated from data in Tables II and VI), the protein accounts for 0.6-1.8% of the total weight, which is also the range frequently quoted in the literature (3) for the protein content of chylomicrons. Zilversmit (7) reports 0.3-0.7% of the total weight of washed chylomicrons as protein. That the difference in the protein contents of the two chylomicron preparations is real is further suggested by the observation that the free sterol content of our chylomicron preparations was 2-3 times that of Zilversmit's, which indicates that a relatively constant protein/sterol ratio was obtained for the membranes of both preparations. The significance of this difference in the protein proportion is difficult to appraise since progressive oiling out of chylomicrons during the washing and high speed centrifugation lowers the total surface-to-volume ratio of the fat particles and would be expected to affect the calculated percentage of protein. Some of the variability in the protein content of different chylomicron preparations may have also resulted from a loss of membrane protein during the sedimentation of the membrane pellet in the centrifuge. It was shown (7) that even within the same preparation, two membrane fractions differed by a factor of 10 in their protein content following a 20-min and 24-hr centrifugation. The longer centrifugation resulted in higher loss of protein due to dissolution.

The possible presence of the extra protein in our chylomicron preparations and concomitant higher amounts of sterol result in somewhat different ratios also for the other membrane components. Thus the sterol/phospholipid ratios, which fall in the range of 0.03-0.07, are only about one half of those reported by Zilversmit (7) for dog and man (0.10), but about twice those reported by him for rats (0.02-0.24). Similarly the protein/phospholipid ratios in our preparations are higher than those that can be calculated from the data of Zilversmit. As pointed out earlier, the protein/sterol ratio, however, remained about the same in both preparations. The extra protein in our samples must therefore have contained more sterol than phospholipid relative to the more tightly bound membrane protein, or it must have had the ability to bind more sterol than phospholipid during the formation of the chylomicron.

The above data are pertinent to the interpretation of the results of studies of chylomicron structure and metabolism (6). Zilversmit (7) has presented an extensive review of the literature relating to his findings. Since in most instances our data compare favorably with those of Zilversmit, no attempt has been made to repeat this discussion here beyond pointing out the minor differences noted between our results and those of Zilversmit.

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A Comparative Study of the Lipids of Globule Membrane and Fat Core and of the Milk Serum of Cows[1]

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ABSTRACT

Nine samples of fresh raw cow's milk were separated into fat globules and milk serum by centrifugation. After destabilization by freezing and thawing, the milk fat globules were resolved into membranes and fat cores. The lipid composition of these structures was compared to that of the surrounding milk serum. Of the total milk fat, 95-98% was in the fat cores, 0.5-1% in the globule membranes and the rest (1.5-4%) in the milk serum. The fat cores contained 88-93% triglyceride, 5.2-9.8% diglyceride, 1.5-7.3% free fatty acid and 0.2-0.4% cholesterol, but no phospholipid. The lipids of the membrane contained 21-44% phospholipid, made up of about equal proportions of phosphatidyl ethanolamine, phosphatidyl choline, and sphingomyelin. The other lipids of the membrane (56-79%) consisted of 83-88% triglyceride, 5.1-10.7% diglyceride, 1-5.1% free fatty acid and 0.4-1.9% cholesterol. The milk serum contained 30-45% phospholipid divided about equally among phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin. The rest (55-70%) of the milk serum lipids was made up of 71-83% triglycerides, 4.3-10.1% diglycerides, 8.7-15.7% free fatty acids, and 1.2-8.4% cholesterol. Corresponding phospholipid classes of milk serum and globule membranes had identical fatty acid compositions. The triglycerides and diglycerides of the globule membranes possessed increased proportions of palmitic and stearic acids in comparison to the glycerides of the fat cores.

INTRODUCTION

MILK FAT GLOBULES are believed (1) to be enwrapped in a surfactant membrane which consists primarily of protein and phospholipid. In several instances (2-4) these membranes have been isolated and the major components determined. This isolation of fat globule membranes of well-defined composition suggests a certain structural rigidity comparable to that found in other biological membranes. Although the presence of a unit membrane in the fat particles has been excluded (5), the binding forces responsible for such rigidity have not been established. An analogous situation now appears (6, 7) to exist in chylomicrons.

If the structure and composition of both particle types depended solely upon noncovalent interactions of the type governing emulsion stability (8), then the composition of the globule membranes should bear a definite relationship to the composition of the respective surrounding serum. Detailed analyses (6, 7) of the lipids of the chylomicrons and the lymph serum do not exclude the possibility of simple emulsification.

In the present study, fresh samples of raw bovine milk have been analyzed under the conditions previously employed for work with chylomicrons. The data obtained confirm and extend earlier observations and demonstrate that, despite large differences in particle size, chylomicrons and milk fat globules possess common chemical features.

MATERIALS AND METHODS

Fresh samples (0.1-0.9 liter) of raw bovine milk were obtained from 6 herds of Holstein cows. Three of the samples were from late summer and 3 from winter feeding. In addition 3 samples of pooled raw cow's milk were obtained from a local dairy.

All reagents and solvents were of Fisher Certified Reagent grade and, with the exception of the petroleum ether, were used without purification. Petroleum ether (bp 30-60C) was redistilled and the fraction boiling in the range 40-56C was used.

Fractionation of Milk

Samples of raw milk (100-200 ml) were divided into fat globules and milk serum by

[1]Taken in part from a PhD thesis submitted by T. C. Huang to Queen's University, Kingston, Canada in April, 1965. Presented in part at the 47th Canadian Chemical Conference and Exhibition held in Kingston, Canada, June 1-3, 1964.

centrifugation at 36,000 g for 1 hr at 5C. The milk fat globules were washed once with 0.9% NaCl and then resolved into the fat cores and globule membranes by freezing-thawing and centrifugation (7). The milk serum was recentrifuged once at 36,000 g for 1 hr at 5C and any residual fat globules removed and discarded.

Separation of Globule Membranes and Fat Cores

The method of preparation of milk fat globule membranes was based on that described by Cole et al. (4), which we had successfully adapted to the preparation of the chylomicron membranes (7). Unlike smaller membranes of the minute chylomicrons, membranes of the large milk fat globules were readily sedimented (36,000 g for 1 hr). Furthermore, the lysis of the milk fat globules was usually complete after a single cycle of freezing and thawing. These observations agree with those of Cole et al. (4) and establish a high degree of reproducibility for the method.

Methods of Lipid Analysis

The total lipids from whole milk, milk serum, and milk fat globule membranes were extracted either by warm chloroform-methanol (2:1, v/v) or ethanol-diethyl ether (3:1, v/v) at 35-40C. The denatured and apparently completely precipitated protein was removed by centrifugation. It was washed once with alcohol, twice with diethyl ether, and dried in vacuo to constant weight. The protein weights thus obtained were used for subsequent surface area calculations (9). The lipid extracts were evaporated to dryness, taken up in chloroform, and separated into neutral lipids and phospholipids by chromatography on silicic acid with chloroform and methanol, as described by Bowyer et al. (10). The neutral lipids and phospholipids were then resolved into their chemical classes by thin-layer chromatography using the techniques described for work with the chylomicron lipids (7). Previously described methods (7) were also used for preparation and gas chromatography of the fatty acid methyl esters, free sterols, and for the gas chromatography of intact triglycerides.

RESULTS AND DISCUSSION

Table I indicates the distribution of the milk fat among the milk serum, globule membranes and fat cores. The fat core contains the bulk (95-98.7%) of the milk lipid. The milk serum contains 0.8-3.35% of the total milk lipid, with some possible difference between the winter (0.8-1.4%) and summer (1.83-3.35%) milks. The globule membrane contains 0.5-2.2% of the total milk lipid, again suggesting a difference between winter (0.4-0.5%) and summer (1.85-2.17%) milks. Similar separations into globule membranes and fat cores of 3 pooled samples of raw bovine milk gave an average of 98% of lipid in the fat core, 0.5-1% in the globule membrane, and 1-1.5% in the milk serum. These proportions for the globule membrane lipids agree closely with the average of 0.6-1.2 g of membrane lipid material per 100 g of fat reported by Rimpila and Palmer (2) and Jenness and Palmer (3). The somewhat larger proportion of membrane lipid in the summer milk may be due to greater proportion of small diameter particles in these milk samples (see Ref. 11 for discussion of factors affecting particle size). Since the radius of the particle appears in the second power in the

TABLE I

Distribution of Total Lipid After Destabilization of Milk Fat Globules[a] (g/1000 ml milk)

Herd[b]	Total lipid		Fat core		Globule membrane		Milk serum	
	(g)	%	(g)	%	(g)	%	(g)	%
				Winter milk				
1	31.66	100	31.12	98.6	0.18	0.56	0.26	0.83
2	40.77	100	40.25	98.7	0.21	0.5	0.32	0.8
3	34.83	100	34.19	98.1	0.15	0.4	0.49	1.4
				Summer milk				
4	21.34	100	20.26	95.0	0.36	1.7	0.71	3.4
5	34.1	100	32.69	95.5	0.63	1.9	0.75	2.2
6	29.18	100	28.01	96.5	0.63	2.2	0.53	1.8

[a] All results are expressed as the mean of duplicate analyses.

[b] The actual volumes of milk taken for analyses were: Herd 1 (940 ml), Herd 2 (650 ml), Herd 3 (600 ml), Herd 4 (100 ml), Herd 5 (100 ml), and Herd 6 (100 ml).

TABLE II

Lipid Composition of Milk Fat Globules and Milk Serum[a]
(% Total Lipid of Fraction)

Herd	Fat core		Globule membrane		Milk serum	
	PL[b]	NL[c]	PL[b]	NL[c]	PL[b]	NL[c]
			Winter milk			
1	0	100	33.7	66.3	44.8	55.2
2	0	100	42.8	57.2	32.9	67.1
3	0	100	44.5	55.5	30.8	69.2
			Summer milk			
4	0	100	33.0	67.0	34.3	65.7
5	0	100	21.6	78.4	36.6	63.4
6	0	100	23.0	77.0	30.9	69.1

[a] Milk samples as described in Table I.
[b] Phospholipids.
[c] Neutral lipids plus free fatty acids.

expression for the surface area ($4\pi r^2$), a slight change in the particle size would be magnified in the surface-to-volume ratio. The size of milk fat particles in a single sample may vary from 1μ to 7μ (11). Changes in the ratio of surface area to volume may also be caused by aggregation during centrifugation as demonstrated for chylomicrons (6).

Table II lists the amounts of phospholipids and other lipids in the fat core and membrane of the milk fat globule and in the milk serum, respectively. As noted for the chylomicrons (7) the cores of the fat particles contain no phospholipid. Apparently all the phospholipid present in the globules is held at the oil-water interface in association with the protein. The proportion of the phospholipids in the membrane varied from 33-45% in the winter milks and from 21-33% in the summer milks. According to Palmer et al. (2,12), the fat globules of washed cream contain 200-400 mg of phospholipid per 100 g of fat; while Heineman (13) quotes an average value of 300 mg/100 g of fat. Mulder et al. (14) claimed a much higher value (600 mg/100 g of fat), but it was derived from the difference between the total milk phospholipids and the skimmed milk phospholipids. According to Mulder et al. (14) about 60% of the total milk phospholipid was present in the surface layer of the fat globule, averaging about 30% of the total membrane lipid. Our data for these lipids are also in this range. The significance of the difference between the winter and summer milks remains uncertain, although speculation could again be made on the basis of a possible difference in the average particle size. The globule membranes from our dairy samples, although two of them were from winter milk, also contained about 30% of the phospholipid.

TABLE III

Composition of Phospholipids of Milk Fat Globule Membrane and Milk Serum[a]

Herd	Milk Fraction	Percentage of total phospholipid		
		Cephalin[a]	Lecithin	Sphingomyelin
		Winter milk		
1	Membrane	30.5	37.2	32.3
	Serum	27.6	37.7	34.7
2	Membrane	32.8	39.6	27.6
	Serum	27.5	41.8	30.7
3	Membrane	31.8	29.2	39.0
	Serum	26.7	35.3	38.0
		Summer milk		
4	Membrane	29.9	45.5	24.6
	Serum	33.0	42.8	24.2
5	Membrane	25.2	45.6	29.2
	Serum	31.6	49.3	19.1
6	Membrane	39.8	37.5	22.7
	Serum	31.8	42.2	26.0

[a] Milk samples as in Table I.

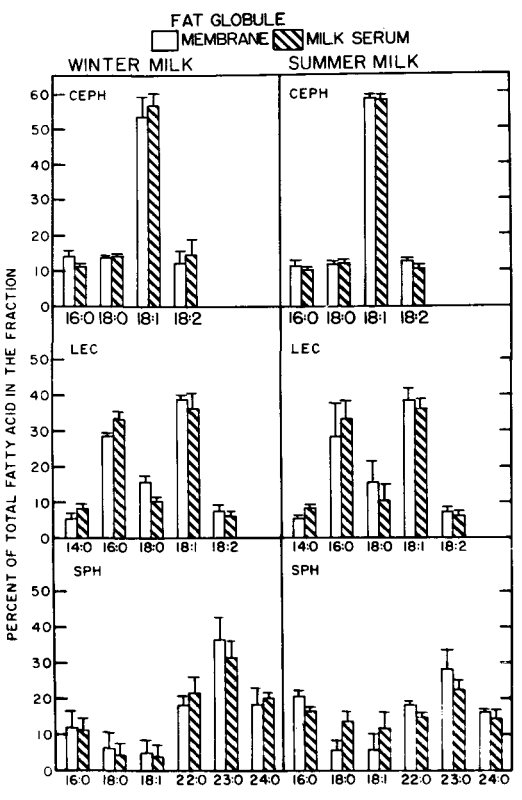

FIG. 1. Fatty acids of individual phospholipids from milk serum and milk fat globule membrane (wt %). Cephalin represents phosphatidyl ethanolamine plus any phosphatidyl serine. Fatty acids identified by the total number of carbons: total number of double bonds. T lines indicate maximum values for the herds in each group.

Phospholipids

Table III gives the composition of the phospholipid classes in the membranes and the milk sera. In all samples approximately equal proportions were found for phosphatidyl ethanolamine (25-33%), phosphatidyl choline (29.2-49.3%) and sphingomyelin (19.1-39.0%); although, there was somewhat more of the phosphatidyl choline in both the serum and the membrane of some of the milk samples. The limited material readily handled on the thin-layer plates did not permit the detection of the cerebrosides and the phosphatidyl inositols. Furthermore, because of incomplete resolution of phosphatidyl ethanolamine and phosphatidyl serine, the estimate for the phosphatidyl ethanolamine includes the serine derivative which accounts for about 8% of the total phospholipid and possesses a fatty acid composition nearly identical to that of the ethanolamine analog (15). These data on the major phospholipids are in agreement with those of Payens (16), who reported milk phosphatides to contain 30% lecithin, 45% cephalin and 25% sphingomyelin, as well as with the comparable estimates of Smith and Lowry (17) and Patton et al. (18). Various methods have been used in the past to measure the milk phospholipids. Morrison et al. (15) have summarized the reports and conclude the approximate amounts of the major components of the phospholipid fraction of milk lipids to be 3% ceramide monohexoside, 3% ceramide dihexoside, 30% phosphatidyl ethanolamine, 1% phosphatidal ethanolamine, 8% phosphatidyl serine, 5% phosphatidyl inositol, 28% phosphatidyl choline, 3% phosphatidal choline and 19% sphingomyelin. Except for our somewhat higher estimates for sphingomyelin in the membrane samples from winter milk, the present values correspond rather closely to these. No estimates, however, have been attempted for the cerebrosides, the phosphatidyl inositols as well as the plasmalogen components of the lecithins and cephalins.

Fig. 1 gives the fatty acid data derived by GLC for the individual phospholipid classes of the milk fat globule membrane and the milk serum. The minimum and maximum concentrations are graphically represented for both winter and summer milks. No statistically significant differences, however, were observed between the two milk types. Also statistically insignificant were the differences in the fatty acid compositions of corresponding classes of phospholipids from the milk serum and the globule membranes. Patton et al. (18) had previously claimed that the membrane phospholipids were identical to the phospholipids of the milk serum but their impressions were not substantiated with analyses of fatty acids. The present finding of an apparently identical phospholipid composition for the membranes and the milk sera would be anticipated if the phospholipids of the milk globule membranes were free to participate in the type of exchanges which Minari and Zilversmit (19) have reported to occur between lymph chylomicrons and plasma.

The cephalins (phosphatidyl ethanolamine plus phosphatidyl serine) were the most unsaturated phospholipid class. Oleic acid accounted for 49–60.7% of the total acid in the milk serum and for 53.8–60.6% of the total acid in the globule membrane cephalins; while linoleic acid made up 8.4–14.2% of the total in the serum and 9.7–18.7% in the membrane. Palmitic and stearic acids occurred in nearly

equal concentrations in both cephalin sources and each accounted for 10.5–15.7% of the total. Small amounts of myristic, palmitoleic and linolenic acid totalling 2.6–7.8% were also detected but these were not included in the tabulations. The values for the major fatty acids of these phospholipids are very close to those recently reported by Morrison et al. (15) for the phosphatidyl ethanolamine and phosphatidyl serine components of spray-dried buttermilk powder. They also demonstrated that the phosphatidyl ethanolamine fraction was generally similar to phosphatidyl serine in total fatty acid composition, although the latter contained a higher proportion of saturated acids.

The lecithin fractions from both serum and membrane had oleic (33–42%), palmitic (28.2–39%) and stearic (10–22%) acids as major components. In addition to these, 5–10% each of myristic and linoleic acids were also found and there were traces (0.1–5%) of lauric, myristoleic, pentadecanoic, palmitoleic, and linolenic acids. The latter were not tabulated. These values are again remarkably close to those of Morrison et al. (15).

The sphingomyelin fractions consisted primarily of saturated fatty acids with 18.9–40% of the total contributed by tricosanoic, 13–27% by behenic, 12.8–22% by lignoceric, 6–10% by stearic and 8.4–21% by palmitic acid. There was 5–18% of oleic acid and traces of other common fatty acids. This peculiar fatty acid composition of the sphingomyelin fraction agreed fully with the findings of Morrison et al. (15) for the spray-dried buttermilk.

The present data and those reported for whole milk phospholipids are therefore in essential agreement. This attests further to the probable identity of the phospholipids of the milk serum and the globule membrane and suggests that this composition remains relatively constant. Whether this identity results from a specific enzymic synthesis from a common lipid pool, or represents the requirements of a specific physicochemical system, remains to be established.

The work of Hawke (20) and Morrison et al. (15) has shown that there is a general tendency for the saturated fatty acids to be concentrated in the α-position of the phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl choline molecules. Exceptions were noted for myristic acid in lecithin, which was largely in the β-position, and oleic acid in phosphatidyl ethanolamine, in which it was almost evenly distributed. Therefore, there must be a significant proportion of molecules containing two saturated acyl groups in phosphatidyl choline. Saturated phosphatidyl ethanolamine and phosphatidyl serine molecules might also exist. Subsequent studies by Blank et al. (21) have confirmed this prediction and have shown that milk lecithins are unique in nature in containing significant amounts (12.7%) of fully saturated lecithins. This finding is interesting in view of the presence of large amounts of high melting triglycerides in the milk globule membranes.

Recent evidence indicates that the milk phospholipids may be, to a large extent, the breakdown products of labile glycophospholipid and proteolipid complexes (22).

Neutral Lipids and Free Fatty Acids

As shown in Table II, both the fat globule membrane and the milk serum contained 55–69% nonphosphatide lipid. Those of the membrane have been collectively referred to in the past as high melting triglycerides (23).

TABLE IV

Composition of Neutral Lipids of Milk Fat Globules and Milk Serum[a] (Percentage total neutral lipid)[b]

Herd	Steryl ester			Free sterol			Triglyceride			Free fatty acid			Diglyceride		
	C	M	S	C	M	S	C	M	S	C	M	S	C	M	S
Winter milk															
1	0.02	0.2	2.3	0.23	1.2	0.8	92.0	85.2	83.1	2.3	2.7	8.7	5.4	10.7	5.1
2	0.01	0.1	0.9	0.16	0.4	0.3	90.3	88.4	77.9	3.7	3.2	10.8	6.0	8.0	10.1
3	0.01	0.3	2.5	0.20	1.4	0.7	93.2	92.2	79.8	1.5	1.0	12.6	5.2	5.1	4.3
Summer milk															
4	0.04	0.3	0.9	0.40	2.6	6.8 ?	82.5	83.5	76.0	7.3	3.1	11.1	9.8	10.5	5.2
5	0.03	0.3	0.8	0.30	1.9	5.2 ?	89.2	85.5	76.1	3.2	5.1	14.2	7.3	7.2	3.7
6	0.02	0.5	3.3	0.30	1.9	5.1 ?	88.0	85.0	71.3	3.9	3.4	15.7	7.8	9.2	4.6

[a] C, fat core, and M, membrane of milk fat globule. S, milk serum.

[b] Original weights as given in Table I.

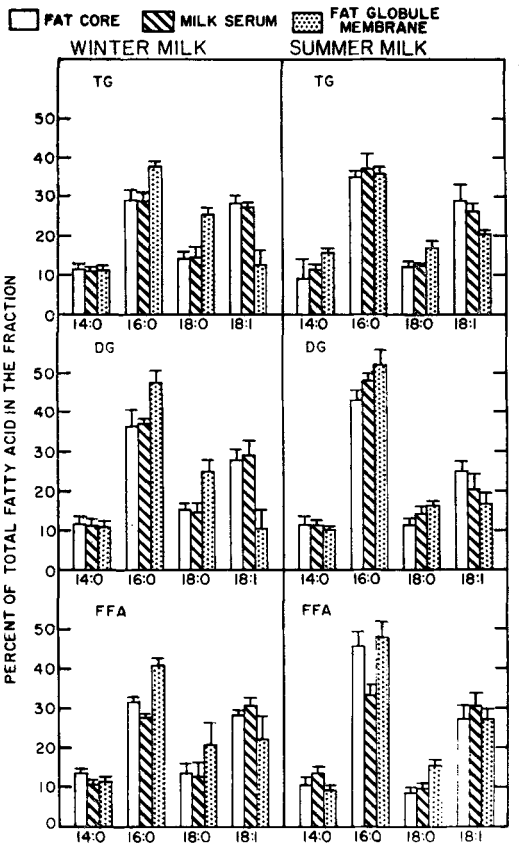

FIG. 2. Fatty acids of neutral lipids of milk fat globules and milk serum. Legends as in Fig. 1.

Careful examination of the nonphosphatide fractions from both the serum and the globule membrane in this study has shown that they contain several lipid classes. Table IV gives a detailed account of the chemical classes of the nonphosphatide lipids found in the fat globule membrane, milk serum and the fat core. The triglycerides make up the bulk (71–92%) of the nonphosphatides.

The largest amount of steryl ester was present in the milk serum where it accounted for 0.9–3.3% of the nonphosphatide lipid; while the fat globule membrane (0.1–0.5%) and the fat core (0.01–0.04%) contained proportionally less significant amounts. Free cholesterol contributed an average of 1–2% of the total neutral lipid of the globule membrane, but values of the order of 0.5% were occasionally found. The fat cores contained 0.16–0.3% of free sterol. The free sterol proportion in the milk serum varied according to the season. While the winter milk contained 0.28–0.7% of the total nonphosphatide in the form of free sterol, it made up 5.1–6.8% of the total in the milk serum from summer milk. Since identical analytical techniques were used it is not immediately obvious why these values should have differed as much. A later set of 3 samples of pooled milk gave values of 1.2–3.2% which are more consistent with the proportions of free sterol found in the membrane neutral lipids.

Free fatty acids made significant contributions to all milk fractions of most samples. The largest amounts of free acids were found in the milk serum, accounting for 8.7–15.7% of total nonphosphatide lipid. The proportions of free fatty acids in the fat core and the globule membrane were comparable and amounted to 1–5%. In addition to these lipid classes, variable amounts of diglycerides (3.7–10.7%) were found which occurred to about the same extent in all milk fractions. Free fatty acids and diglycerides have been identified in freshly churned butterfat (24) and have been assumed to have originated in the milk. Except for the estimates of Thompson et al. (23) of the lipid classes of the globule membrane, no other accounts are available regarding the nonphosphatide composition of other milk fractions. They identified and measured the following membrane constituents (as per cent of membrane lipid): cholesteryl ester (0.79%), free fatty acids plus glycerides other than high melting glycerides (6.3%), diglyceride (8.14%) and monoglyceride (4.66%). In addition to these, Thompson et al. (23) also determined free cholesterol (5.17%), carotenoids (0.45%), and squalene (0.6%), as per cent of the total membrane lipid. Since it was shown in the present study that the membrane contains about 30–40% phospholipid, it can be seen that the estimates of Thompson et al. (23) are somewhat higher than our average values. Particularly great is the discrepancy between the estimates for the free sterol. Furthermore, no monoglyceride at all was found in the present membrane preparations. No attempt of course was made to measure the carotenoids or squalene in our samples. Parallel analyses of the chylomicron lipids (7) have shown a nearly identical composition and distribution for the various nonphosphatide lipid classes among the three phases of the lymph, although there was little steryl ester in the chylomicron membrane.

Fig. 2 gives the composition of the fatty acids of the triglyceride, diglyceride and free fatty acid fractions of the fat cores, globule

membranes and milk sera. The fatty acids of the triglycerides of the milk serum and the fat core are nearly identical, but there are minor differences between the summer and winter milks. Thus the summer milk contains relatively more palmitic and less oleic than winter milk. The triglycerides of the membranes of the winter milk contained proportionally more of palmitic and stearic and less oleic acid than the triglycerides of the corresponding fat cores and the milk serum. The fatty acids of the membrane triglycerides of summer milk were comparable to those of the winter milk, except for a slightly greater proportion of oleic acid and corresponding decrease in the saturated acids of the summer milk. Possibly because of the factor of grazing, the summer milk contains more oleic acid in the glycerides. The differences between the fatty acids of the triglycerides of the fat cores and the globule membranes have been deliberately suppressed in this comparison, since any fatty acids with less than 14 carbons have been excluded from the tabulation. It was shown that the globule fat contained 3.9% butyric and 1–2% each of caproic, caprylic, capric and lauric acids. None of these were present in the triglycerides of the globule membranes. The molecular weight distribution of the globule fat triglyceride was therefore very similar to that previously reported for butteroil (25), while that of the membrane triglycerides approached that demonstrated for the less volatile distillates of this oil (26). The present values for the saturated fatty acids of the membrane triglycerides are considerably below others reported. Thus, Thompson et al. (23) found that palmitic acid alone accounted for 56% of the total fatty acid of the high melting triglyceride. It should be noted, however, that the membrane triglycerides previously studied were usually purified further and recrystallized. Under such conditions an additional increase in the proportion of the saturated triglycerides would be anticipated. Since no specific physiological or structural basis could be found for the division of the membrane triglycerides into high melting and low melting triglycerides, no distinction between the two was made in the present study.

The finding of triglycerides of a somewhat higher degree of saturation in the milk fat globule membranes than in the core or serum agrees with the findings made on the triglycerides of the chylomicron membranes from both corn oil and butterfat feeding (7). Whether these higher melting membrane triglycerides represent a selective removal of the more saturated members of the mixed glyceride population of the fat core during the freezing and thawing process, or are specifically incorporated into the membranes during the formation of the fat particle, remains to be established. It is possible that the unsaturated fatty acids or glycerides, because of their shape, might not be as easily aligned with the membrane phospholipids and proteins as are the saturated acids or their esters and are therefore not as easily incorporated into the membrane.

An examination of the fatty acid composition of the diglycerides and free fatty acids of the globules and the milk serum reveal that they are nearly identical to those of the triglycerides of the corresponding milk fractions. Whether or not this represents a precursor-product relationship cannot be ascertained from the analytical data. Since the free fatty acids of the membranes contained a greater proportion of oleic acid than did the membrane diglycerides, it could be speculated that both were products of enzymic lipolysis of the membrane triglycerides, during which there was preferential lipolysis of the more unsaturated triglycerides. It is known (27) that cow's milk contains several lipases which are adsorbed and activated at the oil-water interface. The origin of the free fatty acids in the milk serum is uncertain. Although lipolysis may explain it, it has been demonstrated that fresh raw milk is also a very good source of an enzyme for glyceride synthesis. McCarthy and Patton (28) showed that ^{14}C-palmitate could be readily incorporated first into diglycerides and finally into the triglycerides of milk fat incubated with this enzyme.

Replicate analyses of the fatty acid composition of the steryl esters of the 3 milk fractions showed widely divergent values in several instances, and the results are, therefore, not reported here. It would have been interesting to compare the fatty acids of the steryl esters of the milk serum with the acids of the sterol esters of the fat core and the globule membrane. In dog lymph and serum studies (7) the steryl esters of the chylomicron fat cores had fatty acids similar to those of the corresponding triglycerides, while the steryl esters of the lymph serum and blood plasma possessed similar fatty acids.

Relation of Protein to Lipid in Milk Fat Globule Membrane

Comparisons of total lipid and protein in the milk fat globule membranes showed consider-

able variation in their relative proportions. The samples of the summer milk contained 20.2–28.4% of the total globule weight as protein. The samples of the winter milk had nearly double the amount. The former values compare well to those observed for the chylomicrons (7) in which 20–30% of the particle weight was contributed by protein. The chylomicrons, however, have much smaller diameters than the milk fat globules and therefore greater surface area to accommodate the protein. It is hoped that in a future publication we will have the opportunity to relate the amounts of protein and lipid in the membrane to the surface area of the milk fat globule (9).

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Influence of Medium-Chain Triglycerides on Lipid Metabolism in the Chick[1]

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ABSTRACT

The effect of corn oil, coconut oil, and medium-chain triglyceride (MCT, a glyceride mixture consisting almost exclusively of fatty acids of 8 and 10 carbons in length) ingestion on lipid metabolism was studied in chicks. In chicks fed cholesterol-free diets, MCT ingestion elevated plasma total lipids and cholesterol and depressed liver total lipids and cholesterol when compared to chicks receiving the corn oil diet. As a consequence of the opposite effects of MCT ingestion on plasma and liver cholesterol and total lipids, the plasma-liver cholesterol pool was not altered. When cholesterol was included in the diets, dietary MCT depressed liver and plasma total lipids and cholesterol as compared with corn oil, consequently also lowered the plasma-liver cholesterol pool.

The in vitro cholesterol and fatty acid synthesis from acetate-1-^{14}C was higher in liver slices from chicks fed MCT than in those from chicks fed corn oil. The percentage of radioactivity from acetate-1-^{14}C incorporated into the carboxyl carbon of fatty acids by liver slices was not altered by MCT feeding, indicating that the increased acetate incorporation represented de novo fatty acid synthesis. The conversion of palmitate-1-^{14}C to C_{18} acids was increased in liver of chicks fed MCT, implying that fatty acid chain elongating activity was also increased. Studies on the conversion of stearate-2-^{14}C to mono- and di-unsaturated C_{18} acids showed that hepatic fatty acid desaturation activity was enhanced by MCT feeding. Data are presented on the plasma and liver fatty acid composition of chicks fed MCT-, corn oil-, or coconut oil-supplemented diets.

INTRODUCTION

DIETARY MEDIUM-CHAIN TRIGLYCERIDE (MCT, a fat consisting almost exclusively of caprylic and capric acid) has been shown to depress serum cholesterol levels in the rat (1-5) as well as in several other species (6-8). In contrast to these observations Fisher and Kaunitz (3) found MCT to be hypercholesterolemic for the chick. This observation was confirmed by Whiteside et al. (9), who reported that chicks fed MCT had higher plasma cholesterol levels than did animals fed corn oil. More recently Fisher and Kaunitz (10) reported that MCT influenced Vitamin E deficiency differently in rats and chicks. In the rat MCT was shown to exert a protective effect against the testicular degeneration induced by Vitamin E deficiency whereas, in the chick, this fat enhanced the exudative diathesis resulting from a deficiency of Vitamin E. The mechanism involved in the differential effects of dietary MCT in these two species is not known, but it has been proposed (10) that MCT may have opposing effects on the linoleate requirement and that this, in turn, may be responsible for the contrasting responses to MCT observed in the rat and chick.

Specific metabolic aspects of lipid metabolism have been studied in rats ingesting MCT. Liver slices obtained from MCT-fed rats show a depressed capacity to convert acetate-^{14}C to cholesterol (4,5). The ability of liver slices from MCT-fed rats to incorporate acetate-^{14}C into fatty acids has been reported to be either normal (5) or elevated (4). The in vitro studies have demonstrated an increased fatty acid chain-lengthening and desaturating capacity in liver of MCT-fed rats (5,11). MCT ingestion has also been shown to increase acetate-^{14}C incorporation into adipose tissue fatty acids, and this increased incorporation is apparently the result of de novo synthesis of fatty acids (5).

The studies were undertaken in an effort to gain a better understanding of the metabolic effects of dietary MCT in the chicken. The data show that liver of MCT-fed chicks has an

[1]The principles of laboratory animal care, as promulgated by the National Society for Medical Research, were observed.

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TABLE I

Percentage of Fatty Acid Composition of Various Dietary Fats and the Pre-experimental Diet

Fatty acid[a]	Corn oil	Coconut oil	MCT	Pre-experimental[b] diet
C_{8}:0	—	6.9	55.9	1.1
C_{10}:0	—	6.5	36.3	0.9
C_{12}:0	—	38.6	3.8	2.2
C_{12}:7[c]	0.6	—	—	—
C_{14}:0	0.3	20.8	1.3	1.9
C_{14}:1	—	—	0.4	1.0
C_{16}:0	14.4	13.6	0.8	13.2
C_{18}:0	2.7	3.5	0.4	6.2
C_{18}:1	19.6	6.4	0.5	14.2
C_{18}:2	56.3	2.7	0.6	20.2
C_{18}:3	6.2	0.9	—	7.7
C_{21}:1[d]	—	—	—	11.9
C_{21}:6[e]	—	—	—	12.3
C_{20}:4	—	—	—	4.3
C_{20}:5	—	—	—	3.0

[a]Chain length: Number of double bonds.

[b]Purina Chick Startena.

[c]Unidentified fatty acid with a retention time of 12.7 carbons.

[d]Unidentified fatty acid with a retention time of 21.1 carbons.

[e]Unidentified fatty acid with a retention time of 21.6 carbons.

increased capacity to synthesize, lengthen, and desaturate fatty acids.

EXPERIMENTAL

Male Hy-line white Leghorn chicks were distributed into the various experimental groups of seven or eight chicks on the basis of body weight. The chicks used in these studies had been fed Purina starter prior to the initiation of the experiments. During the course of these investigations the chicks were housed in heated cages with raised wire floors, and food and water were supplied ad libitum. Body weight and food consumption were determined weekly.

The composition of the basal diet was as follows, in g/100 g of diet: assay protein C-1[3], 30.0; glycine, 0.40; DL-methionine, 0.30; vitamin mix[4], 0.2; choline chloride, 0.2; salt mix[5], 5.31; alphacell, 4; corn oil, 2; glucose, 45.39; and lipid supplement, 12. All diets therefore contained 14% lipid, 2% corn oil, plus an additional 12% of corn oil, coconut oil, or MCT[6]. For purposes of comparison the percentage fatty acid composition of the experimental fats and of the lipids of the pre-experiment diet is shown in Table I. Cholesterol, when added to the diet, was substituted for glucose at a level of 2%.

At the termination of the feeding period, blood was collected by cardiac puncture with a heparinized syringe. The animals were killed by cervical dislocation, the livers were quickly excised, and liver slices were prepared from the left lateral lobe with a Stadie-Riggs hand microtome. The remainder of the liver and plasma was frozen for future analyses. Liver slices (100-200 mg) were incubated in a calcium-free Krebs-Ringer bicarbonate buffer (12), pH 7.4, containing per ml 10 μmoles sodium acetate, 0.167 μc acetate-1-^{14}C, and 5 μmoles glucose. Details of the incubation conditions, isolation of radioactive products, and counting procedures have been described (13). The methods used for the isolation, methylation, and gas chromatographic analysis of fatty acids have also been described previously (5).

Fatty acids were decarboxylated by the method of Brady et al. (14), and the evolved $^{14}CO_2$ was collected and counted as described in an earlier publication (15). Liver lipids and liver and plasma cholesterol were assayed as previously described (16,17). Total serum lipids were determined by the method of Huerga et al. (18). The plasma-liver cholesterol pool (17), as calculated, is the sum of total circulating cholesterol [assuming a plasma volume of 7% of body weight (17)] and total liver cholesterol. This is taken to represent an estimate of total body cholesterol. The data were analyzed statistically by the "t" test.

RESULTS

Plasma and Liver Lipids

The effects of the dietary fats tested on plasma and liver lipid levels of chicks fed cholesterol-free or supplemented diets are shown in Table II. In chicks fed cholesterol-free diets, MCT ingestion increased total plasma lipids. Plasma cholesterol values were 16% higher than in chicks fed corn oil; however this increase in plasma cholesterol level could not be established statistically. In contrast to its apparent elevating effect on plasma cholesterol levels, MCT significantly lowered the

[3]Assay Protein C-1, Skidmore Enterprises, Cincinnati, O.

[4]The composition of salt mix, g/100 g of mix: $CaCO_3$, 5.65; $Ca_3(PO_4)_2$, 52.77; K_2HPO_4, 16.96; $MgSO_4$, 2.36; Fe gluconate, 4.23; $ZnSO_4 \cdot 7H_2O$, 0.083; KI, 0.075; $CuSO_4$, 0.038; H_3BO_3, 0.017; $CoSO_4 \cdot 7H_2O$, 0.002; $MnSO_4 \cdot H_2O$, 1.225; NaCl, 16.58.

[5]Composition of vitamin mix, mg/100 g of mix: thiamine HCl, 625; riboflavin, 400; Ca pantothenate, 500; pyridoxine-HCl, 150; biotin, 15; folic acid, 100; p-aminobenzoic acid, 50; 2-methyl-1,4-napthoquinone, 125; vitamin B_{12}, 0.50; and (in g) α-tocopherol acetate, 2.50; inositol, 2.50; ascorbic acid, 6.25; niacin, 3.75; and (in IU) vitamin A acetate, 250,000; vitamin D_3, 23,000.

[6]The MCT used in these studies was supplied by G. S. Knight, Drew Chemical Company, Boonton, N. J.

TABLE II

Body Weight and Plasma and Liver Lipids of Chicks Fed Different Fats

Dietary fat	Final body weight	Plasma		Liver[a]		Plasma-liver
		Total lipids	Cholesterol	Fat	Cholesterol	Cholesterol Pool
	g	mg%	mg%	%	mg/g	mg
	Experiment I[b] (cholesterol-free diets)					
Corn oil	475 ± 12[c]	553 ± 21	104 ± 5	4.8 ± 0.1	3.6 ± 0.2	73 ± 3
Coconut oil	439 ± 11	631 ± 29	120 ± 5	5.0 ± 0.2	3.2 ± 0.2	71 ± 2
MCT	465 ± 14	666 ± 35	121 ± 8	4.6 ± 0.1	2.8 ± 0.1	70 ± 2
Corn oil vs. MCT[d]	ns	$P < 0.025$	ns	ns	$P < 0.005$	ns
Coconut oil vs. MCT	ns	ns	ns	ns	$P < 0.050$	ns
	Experiment II[e] (cholesterol supplemented diets)					
Corn oil	270 ± 12[f]	2274 ± 217	531 ± 52	11.7 ± 0.4	21.0 ± 0.8	285 ± 22
Coconut oil	287 ± 11	2197 ± 192	536 ± 52	10.1 ± 0.3	18.1 ± 0.9	280 ± 18
MCT	281 ± 5	1786 ± 170	402 ± 53	8.1 ± 0.3	11.0 ± 1.2	183 ± 20
Corn oil vs. MCT	ns	ns	ns	$P < 0.001$	$P < 0.001$	$P < 0.010$
Coconut oil vs. MCT	ns	ns	ns	$P < 0.001$	$P < 0.001$	$P < 0.010$

[a]Liver values are expressed on a wet-weight basis.

[b]Experiments I and II were of 4 weeks' and 3 weeks' duration respectively. Mean initial body weights for corn oil-, coconut oil-, or MCT-fed chicks were 128, 119, and 120 g respectively for Experiment I; 73, 71, and 74 g respectively for Experiment II.

[c]Mean for eight chicks ± standard error of the mean.

[d]Probability of the differences being significant; ns = not significant.

[e]Cholesterol (2%) was included in all diets from Experiment II.

[f]Mean for seven chicks ± standard error of the mean.

concentration of cholesterol in liver of chicks (Table II). The inverse effects of MCT on the plasma and liver cholesterol levels essentially balanced each other, and the plasma-liver cholesterol pool (sum of total plasma + liver cholesterol) was identical for chicks fed MCT, coconut oil, or corn oil.

MCT ingestion significantly decreased total liver lipids and cholesterol in chicks fed cholesterol-supplemented diets (Table II, Experiment II). Total plasma lipids and cholesterol levels of chicks fed MCT were also lower than values for animals fed corn or coconut oil, but these differences were not statistically significant. In spite of the lack of statistical significance, these data suggest that the effects of MCT ingestion on circulating lipids differ in chicks fed cholesterol-free or supplemented diets. As a result of the lower plasma and liver cholesterol levels in MCT-fed chicks the plasma-liver cholesterol pool was decreased by 36% as compared with values for corn oil-fed chicks, a difference which was statistically significant. Plasma and liver lipid values were similar for chicks fed coconut or corn oil.

Fatty Acid Composition of Plasma and Liver Lipids

Plasma and liver samples from chicks used in Experiment I (Table II) were taken for the determination of fatty acid composition. The results of these studies are shown in Table III. The major differences observed are an increase

TABLE III

Percentage of Fatty Acid Composition of Liver and Plasma Lipids of Chicks Fed Different Fats (Experiment I)

Fatty acid[a]	Plasma			Liver		
	Corn oil	Coconut oil	MCT	Corn oil	Coconut oil	MCT
C_{10}:0	7.8 ± 1.1[b]	3.7 ± 0.6	2.6 ± 0.6	0.5 ± 0.1	0.5 ± 0.1	1.0 ± 0.2
C_{12}:0	3.4 ± 1.0	4.8 ± 0.5	1.5 ± 0.2	1.4 ± 0.4	3.4 ± 0.8	0.8 ± 0.1
C_{14}:0	8.4 ± 1.2	8.9 ± 0.7	3.4 ± 0.4	1.1 ± 0.2	5.6 ± 0.7	1.8 ± 0.2
C_{16}:0	16.7 ± 0.5	19.9 ± 1.1	26.2 ± 1.0	17.4 ± 1.0	21.9 ± 0.7	25.4 ± 1.2
C_{16}:1	7.0 ± 0.6	5.4 ± 0.6	5.2 ± 0.3	1.3 ± 0.4	2.4 ± 0.2	3.5 ± 0.3
C_{18}:0	8.5 ± 0.8	10.9 ± 1.2	10.5 ± 0.8	17.1 ± 1.8	14.8 ± 1.9	16.5 ± 1.1
C_{18}:1	8.4 ± 0.5	10.0 ± 0.6	12.2 ± 0.6	7.3 ± 0.4	9.3 ± 0.5	12.1 ± 0.3
C_{18}:2	29.8 ± 0.5	24.4 ± 1.9	25.4 ± 0.5	29.6 ± 0.9	21.7 ± 0.9	19.3 ± 0.8
C_{20}:4	9.3 ± 0.5	12.1 ± 1.0	13.0 ± 0.6	24.3 ± 0.7	20.3 ± 0.9	19.8 ± 1.0

[a]Chain length: number of double bonds.

[b]Mean for eight chicks ± standard error of the mean.

TABLE IV

The in vitro Acetate-1-^{14}C Utilization by Liver Tissue of Chicks Fed Different Fats (Experiment 1)

Dietary fat	CO_2	Fatty acid	Cholesterol
	mμmoles incorporated/100 mg tissue/3 hr		
Corn oil	3454 ± 416[a]	872 ± 196	73.7 ± 6.6
Coconut oil	4978 ± 350	791 ± 125	76.3 ± 7.1
MCT	4758 ± 182	1870 ± 257	112.2 ± 7.8
Corn oil vs. MCT[b]	$P < 0.025$	$P < 0.010$	$P < 0.005$
Coconut oil vs. MCT	ns	$P < 0.005$	$P < 0.005$

[a] Mean for seven chicks ± standard error of the mean.
[b] Probability of the differences being significant; ns = not significant.

in palmitate (C_{16}:0) and oleate (C_{18}:1) and a decrease in linoleate (C_{18}:2) in plasma and liver lipids of chicks fed MCT or coconut oil as compared with values for chicks fed corn oil. These changes were not unexpected since corn oil contains substantial quantities of linoleate; however the changes observed were not as striking as might have been anticipated. This relative lack of difference may be the result of the linoleate content of the pre-experimental diet which was high in linoleic acid (Table I). The high level of C_{10}:0 in the plasma of corn oil-fed chicks is noteworthy, as is the lower value observed in plasma of coconut oil or MCT-fed chicks. The significance of these observations is not clear.

The in vitro Utilization of Acetate-1-^{14}C by Liver Slices

The ingestion of coconut oil or MCT significantly increased over-all metabolic activity as measured by the oxidation of acetate-1-^{14}C to $^{14}CO_2$ by liver slices (Table IV). Liver slices from chicks fed MCT also converted more acetate to fatty acids and cholesterol than did slices from corn or coconut oil-fed chicks.

TABLE V

An Estimation of Fatty Acid Elongation by Liver Tissue by Chicks Fed Different Fats (Experiment 1)

Dietary fat	Decarboxylation of fatty acids synthesized from acetate-1-^{14}C	Conversion of palmitate-1-^{14}C to C_{18} acids
	% of radioactivity in carboxyl carbon	% of radioactivity in C_{18} acids
Corn oil	12.8 ± 4.8[a]	3.0 ± 1.1
Coconut oil	14.5 ± 5.5	3.1 ± 1.2
MCT	12.0 ± 4.5	4.2 ± 1.6
Corn oil vs. MCT[b]	ns	$P < 0.050$
Coconut oil vs. MCT	ns	$P < 0.005$

[a] Mean for seven chicks ± standard error of the mean.
[b] Probability of the differences being significant; ns = not significant.

TABLE VI

An Estimate of Fatty Acid Desaturation Activity by Liver Slices of Chicks Fed Different Fats (Experiment 1)

Dietary fat	Stearate-2-^{14}C incorporated into C_{18}:0	C_{18}:1	C_{18}:2
	% of total radioactivity		
Corn oil	89.7 ± 1.2[a]	7.5 ± 0.9	2.8 ± 0.3
Coconut oil	80.2 ± 3.4	13.2 ± 2.3	6.6 ± 1.4
MCT	80.2 ± 1.7	15.9 ± 1.6	3.9 ± 0.3
Corn oil vs. MCT[b]	$P < 0.001$	$P < 0.001$	$P < 0.050$
Coconut oil vs. MCT	ns	ns	ns

[a] Mean for seven chicks ± standard error of the mean.
[b] Probability of the differences being significant; ns = not significant.

The in vitro Fatty Acid Elongation and Desaturation by Liver Slices

Fatty acid chain-elongation activity was evaluated by two techniques, by determining the relative incorporation of acetate-1-^{14}C into the carboxyl carbon of fatty acids and by determining the conversion of palmitate-1-^{14}C into C_{18} fatty acids. The first procedure will show enhanced chain-lengthening activity if de novo synthesis of fatty acids is not proceeding at high rates. However, if de novo rates of synthesis are high relative to chain-lengthening activity, the latter may be masked. The conversion of palmitate-1-^{14}C to C_{18} acids should yield a measure of chain-elongating activity in spite of high rates of fatty acid synthesis. The data in Table V show that the relative incorporation of acetate-1-^{14}C into the carboxyl carbon of fatty acids similar for chicks fed MCT, corn oil, or coconut oil. This observation implies that the observed increase in acetate-1-^{14}C incorporation into fatty acids by liver of MCT-fed as compared with corn oil-fed chicks is largely the result of enhanced de novo synthesis of fatty acids. Chain-lengthening activity is also increased by MCT ingestion, evidenced by the greater conversion of palmitate-1-^{14}C to C_{18} acids by liver of MCT as compared with corn oil-fed chicks.

Liver slices from chicks fed MCT, corn oil, or coconut oil were incubated in the presence of stearate-2-^{14}C, and the ability of the tissue to convert stearate to C_{18}:1 and C_{18}:2 acids was determined. The data in Table VI show that tissue from chicks fed MCT or coconut oil possessed significantly greater desaturating activity than did liver tissue from corn oil-fed chicks.

DISCUSSION

The hypercholesterolemic effect of MCT ingestion suggested by these studies is in agree-

ment with the original observation of Fisher and Kaunitz (3). The inverse effect of MCT on plasma and liver cholesterol concentration in the chick has also been reported by Fisher and Kaunitz (3). Similar shifts of cholesterol from one compartment to another have been observed in chicks fed different natural fats (17) or fatty acids (19). These observations illustrate the fact that changes in plasma cholesterol level do not necessarily imply a change in the total cholesterol pool.

The contrasting effects of MCT on plasma cholesterol levels in the rat and chick are now well established. Plasma cholesterol is increased in the chick (3,9) and lowered in the rat (1-5) as a consequence of MCT ingestion in a cholesterol-free diet. However the results of the present study show that MCT does not elevate plasma cholesterol in chicks fed cholesterol-supplemented diets. The plasma-liver cholesterol pool was decreased in chicks fed MCT in contrast to animals fed coconut or corn oil. This difference may be a reflection of an alteration in cholesterol absorption since it has been shown that the absorption of cholesterol is enhanced by long-chain unsaturated fatty acids (20). This apparently is not the major factor responsible for the difference in the cholesterol pool size between chicks fed corn oil or MCT since the ingestion of coconut oil, a fat containing extremely small quantities of long-chain unsaturated fatty acids, did not decrease the cholesterol pool size. Consequently the mechanism involved in this effect of MCT remains obscure.

The data demonstrate that MCT ingestion by chicks results in increased hepatic fatty acid synthesis, elongation, and desaturation and cholesterol synthesis. In general, these results are similar to those reported for the rat (5). The notable exception is the increase in hepatic fatty acid and cholesterol synthesis. In the rat, MCT feeding depresses cholesterol synthesis whereas in the chick it is enhanced. Fatty acid synthesis is also markedly increased by MCT feeding in the chick, but in the rat it has been reported to remain unchanged (5) or to increase (4).

The chick also responds differently from the rat to dietary MCT with respect to alterations in plasma and liver fatty acid composition (5). Qualitatively, similar changes are observed in the two species, but the rat fed MCT shows greater fatty acid alterations than does the chick. This is particularly true for linoleate, which was much lower in tissues of MCT-fed compared to corn oil-fed rats, whereas in the MCT-fed chick the decrease in linoleate relative to levels in chicks fed corn oil was not as marked. This resistance to decreased linoleate levels may reflect a greater ability of the chick selectively to retain unsaturated fatty acids as suggested by Feigenbaum and Fisher (21).

The data in Table VI show that the ability of chick liver to desaturate stearate-^{14}C is increased by MCT ingestion. These data also show that chick liver is capable of converting stearic acid to a di-unsaturated acid. It should be pointed out that this conversion does not denote linoleate synthesis de novo. Undoubtedly this di-unsaturated acid is similar to that synthesized by rat liver microsomes (22) and is different from linoleic acid ($\triangle^{6,9}$ vs $\triangle^{9,12}$ for linoleate).

It is now well documented that MCT ingestion significantly lowers the plasma-liver cholesterol pool in rats (4,5) but does not alter the size of the pool in chicks although a cholesterol shift from the liver to the plasma compartment is seen in MCT-fed chicks. This difference in pool size may be related to the effect of MCT on cholesterol synthesis, which is stimulated in the chick and depressed in the rat as a consequence of MCT ingestion. More evidence is required before such a concept can be developed, and the present studies, which have been largely descriptive in nature, have uncovered a number of areas which merit further study.

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Analysis of Fecal Neutral Steroids and Bile Acids in Humans on Constant Fat Diet

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ABSTRACT

A method is described for the separation and quantification of fecal neutral steroids and fecal bile acids. The fecal extract is separated into the neutral steroid fraction and bile acid fraction with ion-exchange resin columns. The principal neutral steroids and bile acids are then separated and quantitated by thin-layer chromatography. Values for the fecal neutral steroids, cholesterol, coprostanol and coprostanone and fecal bile acids, deoxycholic acid and lithocholic acid of 5 subjects on a constant fat diet for a 3-week period are presented.

INTRODUCTION

IN MAN THE MAJOR ROUTE of excretion of cholesterol and its metabolites is the intestine. The daily fecal excretion of neutral plus acidic steroids of endogenous origin should approximately equal the daily synthesis of cholesterol when the steady state is reached. This is based on our knowledge of:

a) The conversion of cholesterol to bile acids in the liver and the degradation of neutral and acidic sterols to a multitude of steroidal secondary products within the intestinal lumen (Danielsson) (1).

b) The resistance of the ring structure of cholesterol to degradation within the body or by intestinal microorganisms (2).

A method for the separation and quantification of fecal neutral and acidic steroids was therefore developed in order to answer certain questions concerning the mechanism by which various compounds lower serum cholesterol values. This method employs ion exchange chromatography and thin-layer chromatography (TLC). The ion-exchange chromatography separates the fecal extract into neutral steroids and acidic steroids. TLC then separates the neutral steroid fraction into three main classes typified by cholesterol, coprostanol and coprostanone together with their plant analogues. The acidic or bile acid fraction is similarly separated into the trihydroxy bile acids, when present, and the dihydroxy and monohydroxy bile acids which are normally predominant. These three types of bile acids are determined on the TLC plate by quantitative densitometry.

MATERIALS AND APPARATUS

All solvents, except 2, 2, 4-trimethyl pentane, were glass-distilled before use and were obtained from British Drug Houses Ltd., Poole, Dorset, England.

Column Chromatography

Column I. A glass column of inside diameter 1.3 cm and length 39 cm was fitted with a stopcock, a coarse sintered disc and a reservoir to hold approximately 50 ml. Dowex 50W-X8 cation exchange resin (hydrogen form, approximately 15.0 g) was suspended in 80% ethanol, poured into the column and allowed to settle to a depth of 16 cm above the sintered disc. The resin was then washed with a further 100 ml of 80% ethanol.

Column II. A glass column similar to Column I contained Dowex 1-X2 anion exchange resin (hydroxyl form—approximately 14.0 g). The resin was converted from the chloride form to the hydroxyl form by suspending it in aq. 1 M NaOH. The suspension was poured into the column and allowed to settle to a depth of 13 cm above the sintered disc before washing with 100 ml aq. 1 M NaOH. The column was then brought to neutrality with distilled water before being washed with 100 ml of 80% ethanol.

The columns were then ready for use and were arranged so that the eluate from Column I drained into the reservoir of Column II.

The Dowex resins were obtained from V.A. Howe Ltd., London, England.

Thin-Layer Chromatography

TLC was carried out on 0.25-mm layers of Macherey Nagel MN Kieselgel G-HR on 20 × 20 cm glass plates. A smooth suspension was obtained by shaking the silica gel with distilled water (30 g in 60 ml water for 90 sec for 5 plates) in a stoppered 250 ml conical flask. The plates were spread with a Camag Spreader,

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left overnight at room temperature to dry and then stored in a desiccator until required.

Scanning of Plates

The optical density (OD) of the charred spots was measured with the Joyce Loebl Chromoscan with Thin Layer Attachment. The attachment is suitable for both reflectance and transmittance operation and uses the recording and integrating facilities of the Chromoscan. The sample table on which the thin-layer plate is placed, the recording drum and integrator are controlled by a single switch on the Chromoscan once the Thin Layer Attachment is plugged in. At the end of each run a microswitch automatically cuts the sample drive. There are filter and slit positions in both reflectance and transmittance light paths.

Collection and Drying of Feces

Feces were collected in weighed plastic containers during a period of 3-6 days and stored in a deep freeze until required. The specimens were then weighed and homogenized with distilled water in a weighed homogenizer. A known aliquot of the homogenate was transferred to a tared 250 ml round bottom flask. The flask was attached to an Edwards Freeze Drier Model 10P and the feces were freeze-dried for approximately 24 hr. When dry, the flasks were reweighed and the dried feces removed and transferred to small screw-capped jars. These were then stored in a desiccator until required.

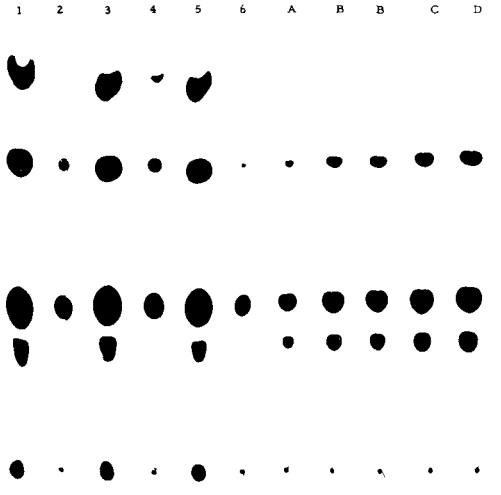

FIG. 1. TLC of fecal neutral steroids and standards. Origin at bottom. From left to right (lanes *1* to *6*) are 6 fecal neutral steroid extracts; lanes *A*, *B*, *C* and *D* contain cholesterol standards (spots nearest origin) 10, 15, 20 and 25 μg, coprostanol standards (second spot from origin) 20, 30, 40 and 50 μg and coprostanone standards (third spot from origin) 10, 15, 20 and 25 μg (Standard B is duplicated). The spot nearest the solvent front in the extracts is a mixture of sterol esters, triglycerides and hydrocarbons.

Extraction of Feces

Approximately 1.0 to 2.0 g of dried feces were extracted with 95% ethanol in a Soxhlet extraction apparatus in a nitrogen atmosphere for 6 hr. The extract was then separated into acidic and neutral steroids by passing it through the Dowex ion-exchange resins.

Neutral Steroid Fraction

The fecal extract was poured into Column I and allowed to percolate through into Column II. When there was approximately 5.0 ml of the extract left in Column I, 50 ml of 80% ethanol was added and the eluate was again allowed to run through into Column II. Column I was allowed to run dry and the combined eluates from Column II were collected. This fraction contained the neutral steroids. The flow rate was approximately 4 to 5 ml/min at this stage.

Bile Acid Fraction

The fecal bile acids were retained on Column II. They were eluted from the column by 250 ml 0.1 M ammonium carbonate in 50% ethanol after washing the column in the following manner according to Kuron and Tennent (3): a) 25 ml 50% ethanol; b) 50 ml distilled water; c) 25 ml CO_2 free water; d) 100 ml aq. 1 M sodium chloride; e) 100 ml aq. 0.5 M ammonium carbonate; f) water to neutrality; g) 25 ml CO_2 free water; h) 25 ml 50% ethanol.

The fecal neutral steroid fraction and the bile acid fraction were then evaporated to dryness on a rotary evaporator (Büchi, Switzerland) and the residues dissolved in acetone, transferred with washings to 15 ml vials and then evaporated to dryness with a stream of nitrogen. The residues were then made up to 3 ml with redistilled acetone and stored in the tightly-capped vials in a deep-freeze until analyzed by TLC.

THIN LAYER CHROMATOGRAPHY

Neutral Steroid Fraction

The neutral steroids were separated into "cholesterol," "coprostanol" and "coprostanone" fractions by TLC on MN-Kieselgel G-HR. The TLC plate was cleaned by initial development in benzene-ethyl acetate (8:1)

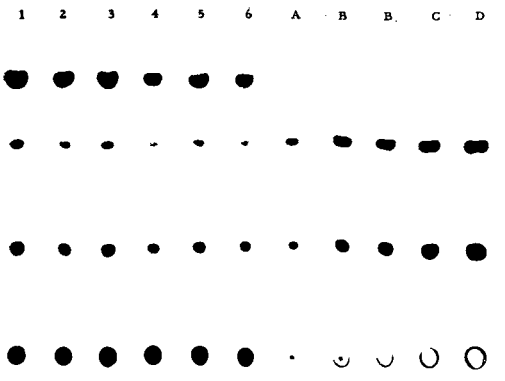

FIG. 2. TLC of fecal bile acids and standards. Origin at bottom. From left to right (lanes *1* to *6*) are 6 fecal bile acid extracts and lanes *A* to *D* contain deoxycholic acid standards (spot nearest origin) 15, 20, 25 and 30 μg and lithocholic acid standards 10, 15, 20 and 25 μg (Standard B is duplicated). The spot nearest the solvent front in the fecal extracts is a mixture of fatty acids.

and allowed to dry at room temperature. The fecal extracts and standards were then applied with a 10 μl Hamilton syringe and the plate was developed in benzene-ethyl acetate (8:1) to a height of 15 cm. Development was carried out at room temperature in a tank lined with filter paper to ensure saturation of the atmosphere. The plate was then allowed to dry. After spraying with 1% potassium iodate in 10% sulfuric acid the plate was placed in an oven at 100C for approximately 20 min. The steroids appeared as brown spots on a white background (Fig. 1).

Bile Acid Fraction

The fecal bile acids were separated into "deoxycholic" acid, "lithocholic acid" (and cholic acid when present) by TLC on MN-Kieselgel G-HR. The bile acid fraction and standards were applied with a 10 μl Hamilton syringe and the plate developed in hexane-diethyl ether-methanol-acetic acid (90:20:3:2). This solvent system caused the fatty acids to move approximately 10 cm in a 15 cm run and left the bile acids at the origin. The plate was then dried and placed in an oven at 100C for 20 min to remove traces of solvent. After cooling, the plate was developed in trimethyl pentane-ethyl acetate-acetic acid (5:25:0:2) (Eneroth, Ref. 4) to a height of 10 cm. This gave a very good separation of cholic, deoxycholic and lithocholic acids and was also effective in separating hydroxy fatty acids from lithocholic acid.

TABLE I

Recovery of Cholesterol and Deoxycholic Acid after Complete Analytical Procedure

Sample	Cholesterol		Deoxycholic Acid	
	Added μg	Recovered μg	Added μg	Recovered μg
1	10.36	10.00	9.50	8.84
2	10.36	10.40	9.50	8.97
Mean	10.36	10.20	9.50	8.91

The plates were treated as before to char the spots (Fig. 2).

After cooling the plates in a desiccator, the optical densities of the spots were measured with the Joyce Loebl Chromoscan with Thin Layer Attachment.

RESULTS

Extraction and Column Chromatography

Recovery experiments were carried out using cholesterol and deoxycholic acids added to dried feces. The fecal specimens, with and without added standards, were subjected to the complete analytical procedure, i.e. solvent extraction under nitrogen, ion-exchange chromatography and thin-layer chromatography. The results were found to be satisfactory as shown in Table I.

The extraction procedure itself was checked by recovery experiments. Deoxycholic acid and cholesterol standards were added to extraction thimbles and the extraction procedure was carried out. After 6 hr, the extracts were evaporated to dryness with a rotary evaporator and the residue dissolved in 3 ml of redistilled acetone. Aliquots were then applied to a thin-layer plate for quantification. The results were again satisfactory as shown in Table II.

Thin-Layer Chromatography

Quantification of steroids and bile acids by measuring the optical density of the spot produced by charring on a TLC plate is dependent on several factors.

1) *A uniformly thick layer of silica gel.* It was found that the Camag spreader gave the most uniformly thick layers. The Desaga and

TABLE II

Recovery of Cholesterol and Deoxycholic Acid by the Extraction Procedure

Sample	Cholesterol		Deoxycholic Acid	
	Added μg	Recovered μg	Added μg	Recovered μg
1	22.70	22.30	11.50	10.95
2	18.30	18.70	10.40	9.80
Mean	20.50	20.50	10.95	10.37

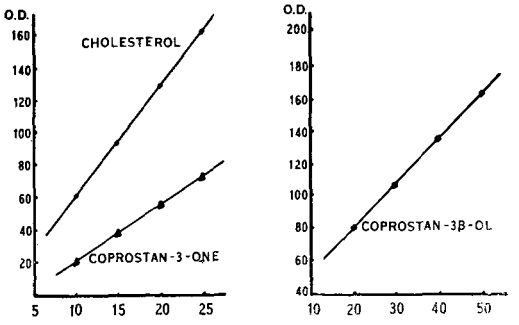

FIG. 3. Standard graphs of cholesterol, coprostanol and coprostanone showing the straight line relationship between OD of the charred spot and quantity of steroid present.

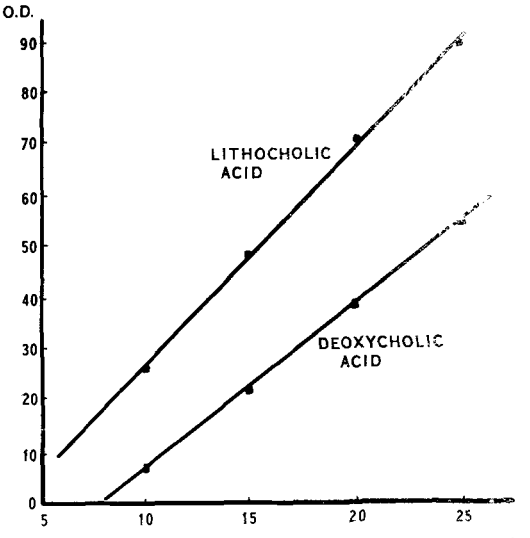

FIG. 4. Standard graphs of deoxycholic acid and lithocholic acid showing the straight line relationship between OD of the charred spot and quantity of bile present.

Shandon spreaders were both tried but were found less suitable for the purpose.

2) *A good separation between well-defined spots.* The techniques described gave well-defined spots and very good separation between the individual spots.

3) *A suitable spray reagent.* It is advisable to produce dark spots on a white background. Various spray reagents were tried, e. g.: a) 10% phosphomolybdic acid in ethanol gave blue spots on a yellow background which was not uniform in optical density. b) 1% ceric sulfate in 10% sulfuric acid gave colored spots on a faintly yellow background. c) potassium dichromate in 80% sulfuric acid gave an uneven grayish background with black specks. d) 35% phosphoric acid gave a background similar to c).

However, it was found that spraying with 1% potassium iodate in 10% sulfuric acid and heating at 100C for 20 min gave dark brown spots on a white background which proved extremely suitable for scanning.

Scanning and TLC Plates with the Chromoscan

It was only after trying various combinations of slit widths, reflectance, transmission and different sizes of cams that we found the best results were obtained using the narrow slit and transmission on the Thin Layer Attachment combined with the largest cam in the Chromoscan. By setting up the instrument under these conditions we were able to obtain a straight line relationship between the amount of steroid or bile acid applied and the OD of the charred spot as recorded by the integration on the Chromoscan (Fig. 3 and 4). The range of standards used was cholesterol and coprostanone 10-25 μg, coprostanol 20-50 μg, deoxycholic acid 15-30 μg and lithocholic acid 10-25 μg.

Recoveries of Neutral Steroids and Bile Acids Using the Scanner

Neutral Steroids. Recovery experiments were carried out by adding known amounts of cho-

TABLE III

Neutral Steroids: Recoveries of Cholesterol, Coprostanol and Coprostanone on TLC Plate with the Scanner

Cholesterol		Coprostanol		Coprostanone	
μg Added	μg Recovered	μg Added	μg Recovered	μg Added	μg Recovered
15	14.8	20	21.3	10	9.9
"	16.3	"	20.3	"	9.9
"	16.3	"	18.1	"	8.4
"	15.8	"	19.6	"	10.5
"	15.8	"	20.5	"	9.0
"	15.5	"	22.6	"	8.0
Mean	15.75		20.41		9.28
S.D.	±0.560		±1.226		±0.975
%	105%		102%		93%

TABLE IV

Bile Acids: Recoveries of Deoxycholic Acid and Lithocholic Acid on TLC Plate with the Scanner

Deoxycholic acid		Lithocholic acid	
μg Added	μg Recovered	μg Added	μg Recovered
15	14.0	15	15.8
"	14.0	"	13.0
"	13.6	"	16.3
"	13.6	"	16.5
"	13.8	"	16.3
"	13.8	"	16.3
Mean	13.8		15.7
S.D.	±0.179		±1.341
%	92%		105%

lesterol, corprostanol and coprostanone to fecal extracts already applied to the TLC plate and then chromatographing the fecal extracts, the fecal extract with the three steroids and a series of standards on the same plate. The results obtained were satisfactory (Table III).

Bile Acids. A similar recovery experiment was carried out but this time deoxycholic acid and lithocholic acid were applied to the extracts. The results obtained were again satisfactory (Table IV).

It was necessary, however, to include a series of standards with each plate as the degree of charring varied from plate to plate even when the temperature of the oven and the time the plate was in the oven were kept constant. This reduced the number of extracts which could be run on each plate to 6.

Tables V and VI show the results of a study in a metabolic ward of 5 patients on a constant fat diet over a period of 5 weeks. The fecal collections were pooled in 7-day periods and the first 2 weeks were discarded to ensure complete adjustment to the diets.

DISCUSSION

Neutral Steroids

The quantitative determination of individual fecal neutral steroids has proved to be very difficult because of their similarities in molecular structures and physical properties. Over the past few years there have been several methods published. For example, the steroids may be precipitated as digitonides followed by regeneration and differential-photometric analysis (Haust and Beveridge) (5) or separated by TLC (Samuel et al.) (6), silicic-acid-impregnated glass paper chromatography (Goldsmith et al.) (7), column chromatography (Bowers et al.) (8), GLC (Eneroth et al.) (9), or a combination of TLC and GLC (Miettinen et al.) (10).

Of these methods only the last two, (9) and (10), measure the individual steroids. The method of Haust and Beveridge (5) depends on precipitation by digitonin; coprostanol is known to be incompletely precipitated by this reagent (Wells and Moore) (11).

The technique described in this report resembles that of Goldsmith et al. (7) in that it measures the OD of the charred spots and that each spot is a mixture of the parent compound and analogous plant sterol metabolites. However Goldsmith et al. found a straight line relationship between 0.6 μg and 1.2 μg, whereas we have found a straight line relationship over a much wider range: cholesterol and coprostanone 10 μg-25 μg and coprostanol 20 μg-50 μg. Our procedure also gives improved separation and a lower level of background absorption.

TABLE V

Subject	Cholesterol mg/24 hr Weeks			Coprostanol mg/24 hr Weeks			Coprostanone mg/24 hr Weeks			Total mg/24 hr Weeks		
	1	2	3	1	2	3	1	2	3	1	2	3
1	82.6	31.7	76.7	159.6	216.4	196.7	69.1	75.4	74.5	311.3	323.5	347.9
2	71.6	54.9	64.5	172.4	155.8	198.7	24.0	12.2	34.4	268.0	222.9	297.6
3	67.2	90.1	178.9	196.5	172.3	155.2	23.5	34.8	50.8	287.2	297.2	384.9
4	35.9	54.3	47.4	186.8	208.9	180.8	34.8	29.3	33.5	257.5	292.5	261.7
5	67.0	54.9	37.7	291.8	345.3	290.2	18.8	26.4	14.2	377.6	426.6	342.1

TABLE VI

Subject	Deoxycholic acid mg/24 hr Weeks			Lithocholic acid mg/24 hr Weeks			Total mg/24 hr Weeks		
	1	2	3	1	2	3	1	2	3
1	59.5	36.1	35.8	50.0	55.7	31.1	109.5	91.8	66.9
2	23.0	23.4	28.0	20.0	19.8	19.7	43.0	43.2	47.7
3	13.3	39.3	34.8	7.3	14.2	5.3	20.6	43.5	40.1
4	8.8	5.5	9.2	9.3	11.1	6.7	18.1	11.1	15.9
5	77.3	68.4	54.1	13.9	15.4	8.3	91.2	83.8	62.4

One disadvantage, however, with this particular method is that it does not differentiate between the plant sterols and parent compound. Thus the "cholesterol" spot may be composed of cholesterol, β-sitosterol, campesterol and possibly other congeners; and, similarly, the "coprostanol" and "coprostanone" spots may represent mixtures of homologues. On an ordinary mixed diet, however, the contribution of plant sterols to fecal neutral steroids is small and relatively constant (9, 12) and therefore this technique is not invalidated. The technique was in fact devised to determine the effect of a hypocholesterolemic drug on fecal excretion of neutral and acidic steroids when the patient was receiving an ordinary diet. The results of the preliminary basal peroid only are published in this article in order to illustrate the method.

Fecal Bile Acids

Bile acids are excreted by the liver into the bile, conjugated with glycine or taurine. As a consequence of bacterial transformation in the intestine the simple mixture of primary bile acids synthesized by the liver (cholic and chenodeoxycholic acids) is excreted as a complex mixture with deoxycholic acid and lithocholic acid the main fecal bile acids (13). It is the complexity of the mixture which has caused so much trouble in the separation and quantification of fecal bile acids.

Various methods have been devised to quantify fecal bile acids. This has usually involved titration of the total bile acid and has not always been successful due to incomplete removal of acidic contaminants. For a recent review see Grundy et al. (14). In the method devised by these authors, the bile acids are isolated by TLC column chromatography and then quantified as the trimethylsilyl ethers of their methyl esters.

More recently Eneroth et al. (15) have utilized column chromatography and TLC and identified the bile acids with GLC combined with mass spectrometry. They have not produced absolute values but from relative proportions they concluded that deoxycholic acid and lithocholic acid were the main components.

Semenuk and Beher (16) have devised a method of direct densitometry of bile acids on thin-layer chromatograms. They used phosphomolybdic acid to locate the spots. We found this spray reagent to be unsuitable due to the non-uniformity of optical density of the background. The amount of bile acid measured by Semenuk and Beher ranged from 0.25 to 4 μg, whereas we determined 10-25 μg.

We decided to estimate the two main groups of fecal bile acids, i.e. the dihydroxycholanic acids and the monohydroxycholanic acids. Deoxycholic acid would be the principal component of the dihydroxy group and lithocholic acid would be the principal component of the monohydroxy group. Cholic acid, which was very rarely found, could be separately determined.

The isolation of the bile acid fraction from the neutral steroid fraction is based on the method of Kuron and Tennent (3). This method according to Grundy et al. (14) results in erroneously high values if titration is used for estimation. We have confirmed this observation and found that it is due to acidic pigments which continually bleed from the ion-exchange resin and which contaminate the bile acid fraction. However, using our TLC procedure involving double development we were able to separate the bile acids from these acidic pigments and also from the fatty acids and hydroxy fatty acids which are present in the bile acid fraction.

Tables V and VI show the results obtained for total and individual neutral steroids and bile acids in three 7-day fecal collections from 5 subjects on an ordinary ward diet, the fat content of which was kept constant. There are considerable intersubject differences both for neutral steroid and bile acids, but there is much less variation in the levels obtained in the same subject over the 3-week period.

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(S)-1,2-Diacyl-3-acetins: Optically Active Triglycerides from *Euonymus verrucosus* Seed Oil[1]

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ABSTRACT

The seed oil of *Euonymus verrucosus* Scop., family Celastraceae, contains more than 90% 1,2-diacyl-3-acetins (monoacetotriglycerides). The constituent triglyceride acids, other than acetic, are the usual long-chain fatty acids.

Thin-layer chromatography (TLC), infrared (IR) and hydrolysis with pancreatic lipase indicate that the acetic acid is esterified exclusively on the outer glycerol carbon atoms. The isolated mixed monoacetotriglycerides exhibit optical rotation caused by asymmetry of the central glycerol carbon atom. Comparison with synthetic products of known configuration shows that the natural material is essentially all (*S*)-1,2-diacyl-3-acetin.

IR, TLC and GLC analyses indicate the presence of monoacetotriglycerides in seven other species of Celastraceae and five species in three other plant families in amounts from 13 to 98%.

INTRODUCTION

ALTHOUGH OPTICALLY ACTIVE SEED OILS are known, their activity results from the presence of optically active fatty acids such as ricinoleic (1), chaulmoogric (1), or 8-hydroxy-5,6-octadienoic (2) acids or of active minor components (1). No natural, unaltered seed oil was known to be optically active because of asymmetry only at the central carbon atom of the glycerol moiety (3) before our preliminary communication (4).

In this paper we include details of isolation and characterization in support of our report (4) of the presence in seed oil of *Euonymus verrucosus* (family Celastraceae) of 1,2-diacyl-3-acetins which are optically active and derive their activity from asymmetry at the β-carbon of the glycerol.[3] Evidence is also presented to show the presence of major amounts of monoacetotriglycerides in oils of 12 other species in 4 plant families; however, the optical activity has been investigated only in *E. verrucosus*.

Acetic acid has long been recognized as a seed oil constituent (5); Gunde and Hilditch (6) in 1938 speculated that the acetic acid in oil from *Celastrus scandens* is esterified with an unknown tetrahydric alcohol. The first report of acetic acid as a natural triglyceride constituent was made by Kaufmann and Keller (7), who established its presence in the seed oil of *Impatiens roylei* (Balsaminaceae). Recently, Bagby and Smith found an asymmetric monoacetotriglyceride in *Impatiens edgeworthii* (8).

METHODS

General

Seeds were either collected from wild plants or purchased from commercial seed suppliers. They were ground and extracted in a Soxhlet apparatus with petroleum ether (30-60C) for 24 hr. Solvent was removed from the oil on a steam bath under a stream of nitrogen.

IR spectra were recorded by Perkin-Elmer Model 137 and 337 spectrophotometers. Samples were either 1% solutions in CCl_4 (1-mm NaCl cell) or films on NaCl discs. Ultraviolet (UV) absorption of ethanol solutions was measured in a 1-cm cell in a Beckman DK-2A spectrophotometer. Observations were made in the region from 215-360 mμ. A Varian A-60 spectrometer was used to measure nuclear magnetic resonance (NMR) in deuterio-chloroform solution containing tetramethylsilane as internal standard.

Methyl esters were analyzed by gas-liquid chromatography (GLC) in a Burrell Kromo-Tog K-5 as described earlier (9) or in an F&M 810 chromatograph equipped with a 10 ft × ⅛ in. stainless steel column packed with 20% LAC-2-R 446 on Celite 545. The injection port was held at 300C and the column at 200C. A flame ionization detector was used. Triglycerides were also analyzed by GLC in the F&M 810 instrument. Conditions were those described by Litchfield et al. (10), except that the samples injected were 0.02 μl as delivered by a Hamilton 7101 syringe.

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[3] For convenience, the 1,2-diacyl-3-acetins will be referred to as monoacetotriglycerides.

Melting points were determined on a Fisher-Johns melting point apparatus. Samples were hydrogenated in hexane in a micro-hydrogenator with 10% palladium on charcoal as catalyst.

Thin-layer chromatography (TLC) of oils and monoacetotriglycerides was performed on 20 × 20 cm plates spread with a 250-μ layer of Silica Gel G impregnated with boric acid. A "sandwich" chamber 1 mm thick was used with a hexane-ether (70:30 v/v) solvent system. Spots were detected by spraying with 0.2% dichlorofluorescein and observing the plate under UV light, by charring with sulfuric acid-dichromate solution or by immersing in iodine vapor.

Analysis for Short-Chain Acids and Glycerol

Separate ½ g samples of *Euonymus verrucosus* oil, soybean oil and a mixture of approximately 50% acetic and 50% formic acids were refluxed for 3 hr with 10 ml 1 N ethanolic KOH. Most of the alcohol was removed by evaporation on a steam bath, water was added to each sample and the solutions were freeze-dried overnight. A portion of the inner flask wall was rinsed with ethanol and the resulting solution was analyzed by GLC in a 20% LAC-2-R 446 column at 200C. The dried solids were placed in individual test tubes, covered with ether and acidified with a minimum of 6 N HCl. The tubes were shaken to extract the free acids into the ether layer. The ether solution was analyzed in an Aerograph A-700 chromatograph equipped with a thermal conductivity detector and a 20 ft × ⅛ in. copper column packed with 20% LAC-2-R 446 on Celite 545. The column temperature was held at 150C.

Isolation and Fractionation of Monoacetotriglycerides

Monoacetotriglycerides were isolated from *E. verrucosus* oil by column chromatography on Adsorbosil-CAB, 100/140 mesh (Applied Science Laboratories, Inc.). One-half-gram samples of oil were applied to 25 g absorbent and eluted with redistilled hexane containing increasing amounts of ether. The separation was monitored by TLC.

Pancreatic Lipolysis

Euonymus oil was hydrolyzed with pancreatic lipase by the method of Mattson and Volpenhein (11). Products were separated by TLC on 20 × 20 cm plates spread with 1-mm layers of Silica Gel G impregnated with boric acid. Plates were developed with hexane-ether (70:30 v/v) in a 1-mm sandwich cell. The recovered lipolysate fractions were analyzed by GLC according to the method described by Tallent et al. (12). Methyl esters were also prepared from each lipolysis fraction and these esters were analyzed by GLC as described above.

Preparation of Synthetic (S)-1,2-diacyl-3-acetin

(*S*)-1,2-diacyl-3-acetin (L-1,2-diacyl-3-acetin) was prepared from chromatographically pure lecithin from egg yolk (13) by the method of Renkonen (14). The lecithin was treated with phospholipase C (Sigma Chemical Company) to give 1,2-diglycerides, which were then acetylated by acetic anhydride in pyridine.

(*S*)-1,2-distearo-3-acetin (L-3-aceto-1,2-distearin) was synthesized essentially by the methods of Baer and Fischer (15) and Sowden and Fischer (16). Hydrogenolysis of the benzyl ether was performed in ethanol with 10% palladium on charcoal as catalyst, and the final product was purified by column chromatography as described above for the isolation of monoacetotriglycerides from *Euonymus* oil.

The products of these reactions are, according to the Cahn-Ingold-Prelog rule (17,18) and the Hirschmann convention (19), (*S*)-1,2-diacyl-3-acetins. The Cahn-Ingold-Prelog rules for glycerides are reviewed by Schlenk (20).

Optical Rotatory Dispersion

Optical rotatory dispersion (ORD) was determined on the monoacetotriglyceride fraction of *E. verrucosus* oil and on the synthetic glycerides in a Cary Model 60 recording spectropolarimeter in a 0.5-dm cell at 25C. A separate portion of the *E. verrucosus* monoacetotriglycerides was transesterified directly in the spectropolarimeter cell with 5% HCl in methanol-hexane (4:1) at 25C. The optical rotation at 350 mμ was recorded as the reaction progressed.

RESULTS AND DISCUSSION

Characterization of Euonymus verrucosus Oil

Seed of *E. verrucosus* contained 49% of oil which differed from common oils in its IR and UV absorption and its behavior in TLC and GLC.

Strong absorption in the IR occurred at 8.1, 7.3 and 9.5 μ, where there is no absorption or much weaker absorption by common oils. The most prominent band was that at 8.1 μ, which has been attributed to acetate groups (21). The band at 7.3 μ is considered to be due to

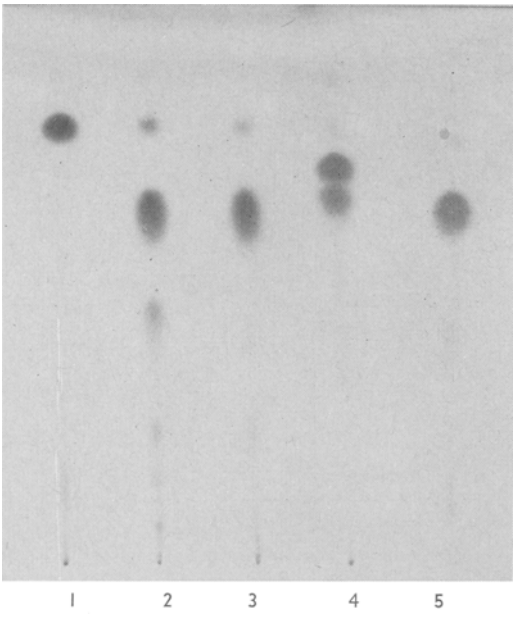

FIG. 1. TLC of acetotriglycerides and oils containing monoacetotriglyceride: (1) Soybean oil (for comparison); (2) *Akebia quinata* oil; (3) *Euonymus verrucosus* oil; (4) acetylated Hormel mixed distearin; (5) hydrogenated monoacetotriglycerides from *E. verrucosus*.

C-H stretching in terminal methyl groups (22) and is increased because three such groups occur in a molecule only about three-fourths as large as the usual triglycerides. The band at 9.5 μ, absent from the spectra of normal oils, is found in α-acetotriglycerides but not in β-acetotriglycerides (23).

UV absorption occurred at 225, 257, 264, 271 and 281 mμ. Maxima at 228, 273 and 280 have been reported for methyl benzoate (ε_{228}=10961) (24). The absorption at 228 mμ is equivalent to that of 2.6% of methyl benzoate, but the actual compound present has not been identified. Benzoic, formic and acetic acids have previously been reported in oils of the Celastraceae (5).

TLC of *Euonymus* oil (Fig. 1) on a plate of Silica Gel G showed little normal triglyceride. The major component of the oil had the same mobility as α-acetodistearin, and there was no component corresponding to β-acetodistearin. Some more polar components remain near the origin.

GLC of the oil, in agreement with TLC, indicated only a trace of components corresponding to those in soybean oil analyzed for comparison. The two major peaks emerged from the column at retention times corresponding to those for trilaurin and acetodistearin and should have the corresponding numbers of carbon atoms in the acid moieties (C_{36} and C_{38}) (Fig. 2). These components could then contain C_2, C_{16} and C_{18} acids (C_{36}) and C_2, C_{18}, C_{18} (C_{38}). These structures are in agreement with GLC of methyl esters prepared from the oil. Of the longer chain acids only C_{16} and C_{18} acids were present. Analysis for volatile acids from the lyophylized soaps revealed only acetic acid; the analysis was not run long enough to detect benzoic acid. Formic acid added as an internal standard formed a distinct peak which emerged after the acetic acid. When a mixture of formic and acetic acids was carried through the saponification and analysis, two peaks occurred that corresponded to the peaks from the sample to which formic acid had been added as an internal standard. Soybean oil, carried through the process, showed no short-chain acids.

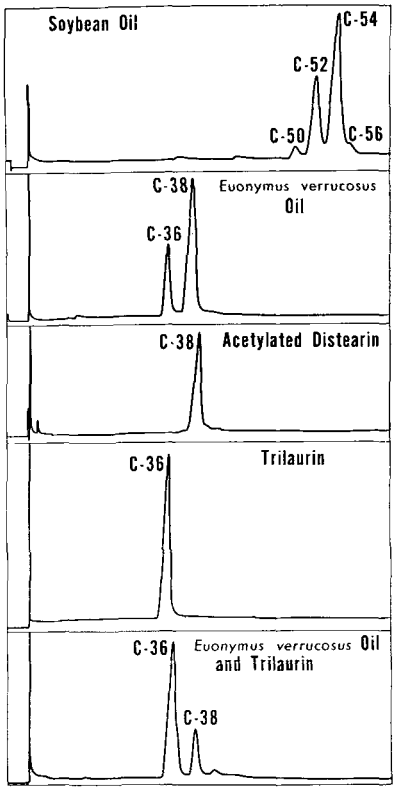

FIG. 2. GLC of *E. verrucosus* oil and of standard triglycerides.

GLC of the alcohol-wash of the flasks in which freeze-drying took place showed only one peak other than that for alcohol. Glycerol itself and the alcohol extract of the soaps from soybean oil gave the same peak.

Pancreatic Lipolysis of Euonymus Oil

Since our material is essentially all α-acetotriglycerides, the pancreatic lipolysis method of determining acyl groups in the β-position is applicable (25). Preparative TLC of the lipolysate of *Euonymus* oil provided the usual products of this procedure and, in addition, two unusual products. One of these migrated in the same region as a monoacetotriglyceride and was subsequently identified as such by TLC and IR. The other migrated between the normal mono- and diglyceride fractions. This second unusual component showed the IR absorption at 7.3, 8.1 and 9.5 μ due to the acetate group. Furthermore, its absorption because of C-H stretching was weaker and that caused by the hydroxyl stronger than the corresponding absorptions of usual diglycerides. After silylation, the component emerged from the GLC column with the silylated C_{18} monoglyceride. Since silyl and acetyl groups seem to contribute equal mobility to partial glycerides in GLC, this lipolytic product must consist of diglycerides containing acetic acid and one long-chain acid (monoacetodiglycerides).

TABLE I

Methyl Ester Composition (Excluding Acetic Acid) of *Euonymus* Oil and Selected Pancreatic Lipolysis Fractions

Acid	Original %	Monoglyceride %	Monoacetodiglyceride %	FFA %
16:0	14	Trace	Trace	29
18:0	4	Trace	Trace	8
18:1	40	45	44	36
18:2	40	50	52	25
18:3	2	4	4	1

Analyses of the methyl esters prepared from selected lipolysis fractions are shown in Table I. As expected, the free acid fraction, derived from the α-carbon atoms of the glycerol, is rich in saturated acids and the monoglyceride fraction has essentially no saturated acids. The excellent agreement between the proportion of the long-chain acids of the monoacetodiglyceride fraction (acetic acid was lost in the routine ester preparation) and those of the monoglyceride fraction confirms that the analyses are indeed representative of the acids combined at the β-position.

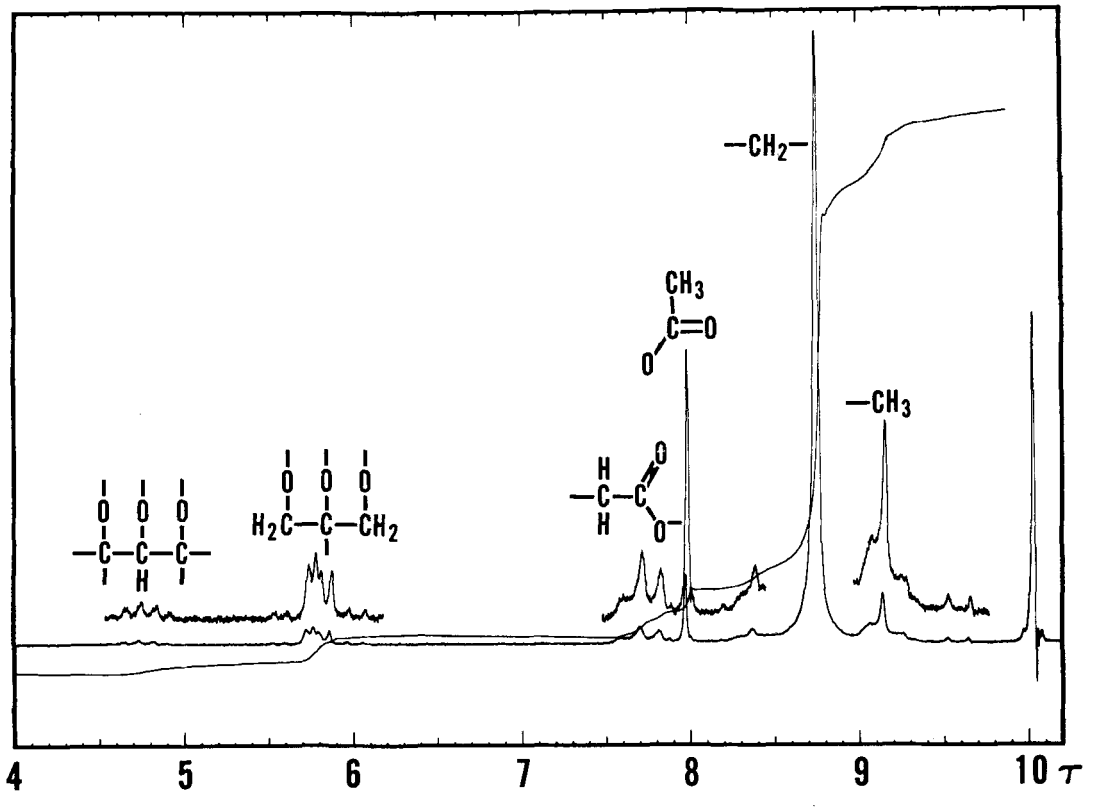

FIG. 3. NMR of hydrogenated *Euonymus* acetotriglycerides.

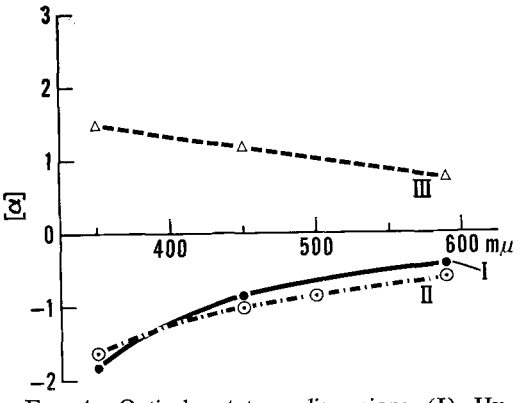

FIG. 4. Optical rotatory dispersion: (I) Hydrogenated *Euonymus* monoacetotriglycerides in hexane; (II) synthetic (*S*)-1,2-distearo-3-acetin in hexane; (III) monoacetotriglycerides from lecithin in methanol: hexane (4:1).

Fractionation of Euonymus Oil

Chromatography of *Euonymus* oil on an Adsorbosil column gave about 90% of the oil as a fraction that showed only one spot by TLC and corresponded to the major component in the TLC of the whole oil. GLC of this fraction gave only the C_{36} and C_{38} peaks observed in a similar analysis of the whole oil. Absorption bands of 7.3, 8.1 and 9.5 μ were the outstanding features of the IR spectrum. Absence of maxima in the UV indicated that no benzoic acid accompanied this fraction.

After hydrogenation of a portion of the fraction, NMR peaks were at 9.1 τ (long-chain terminal methyl protons), 8.7τ (methylene protons other than those α to the carboxyl group), 8.0 τ (acetate protons), 7.8τ (methylene protons α to the carboxyl group), 5.8 and 4.8 τ (glycerol protons) (Fig. 3). Peak ratios were those expected for a saturated monoacetotriglyceride; i.e., for 3 acetate protons there were 5 glycerol protons and 6 long-chain terminal methyl protons.

The hydrogenated fraction was essentially identical to synthetic α-acetodistearin in its IR spectrum, mobility in TLC and GLC and its bands in NMR and their quantitation.

Optical Rotatory Dispersion

Curve I (Fig. 4) represents ORD of the hydrogenated monoacetotriglycerides from *E. verrucosus* oil. The unsaturated *Euonymus* monoacetotriglycerides gave essentially the same curve. Curve II represents the synthetic (*S*)-1,2-distearo-3-acetin prepared from D-mannitol. Sowden and Fischer (16), who first prepared this material, reported 0.0° specific rotation at the D line for their product. The observed rotation of our sample at the D line was only –0.010° (equivalent to a specific rotation of –0.57°) but modern instrumentation permits reliable measurement of rotation of this magnitude. Hydrogenated acetotriglycerides prepared from lecithin had specific rotations in hexane essentially the same as Curve I. The small amounts of C_{20} and C_{22} acids from the lecithin have little effect on optical rotation of the acetotriglycerides at the wavelengths used.

Absence of chiral centers other than that in the glycerol was confirmed by direct transesterification of the natural optically active triglyceride in the spectropolarimeter cell. The methanol-hexane solvent caused reversal in the sign of optical rotation of the natural triglycerides as well as in the monoacetotriglycerides prepared from lecithin (Curve III, Fig. 4). Such reversals due to solvent effects are not unique to these materials (26,27). After standing at room temperature 15 min (the time required for the solution to become clear) the specific rotation of the reaction at 350 mμ was +2.3°. The specific rotation progressively decreased to 1.8° at 30 min, 0.6° at 45 min and 0.0° at 195 min. At this last time, TLC showed the presence of only methyl esters

TABLE II

GLC Analysis of Oils Containing Monoacetotriglycerides

Source	Monoaceto-triglyceride %	Normal triglyceride %	Other %
Celastraceae			
Celastrus orbiculatus Thunb.	84	4	12
Celastrus scandens L.	81	9	9
Euonymus alatus (Thunb.) Sieb.	98	2	....
Euonymus europaeus L.	81	15	4
Euonymus latifolius (L.) Mill.	80	8	12
Euonymus verrucosus Scop.	96	2	2
Gymnosporia royleana M. Laws.	69	24	8
Maytenus illicifolia Mart.	68	11	21
Lardizabalaceae			
Akebia quinata Decne.	87	8	5
Akebia trifoliata (Thunb.) Koidz.	91	9	....
Ranunculaceae			
Adonis aestivalis L.	18	82	....
Rosaceae			
Sorbus aucuparia L.	32	68	....
Sorbus mougeoti Godr. and Soy.-Will.	13	87	....

and glycerol in the solution. The disappearance of optical activity with the destruction of the glycerol chiral center, in conjunction with other data reported, precludes the presence of other chiral centers such as those previously reported in triglycerides optically active because of asymmetry in the fatty acid moieties (1-3).

The acetoglyceride is therefore all, or essentially all, (*S*)-1,2-diacyl-3-acetin. In the biosynthetic processes, the enzyme system must distinguish between the 1- and 3-positions. This specificity has been demonstrated in other triglycerides by Brockerhoff and Yurkowski (29) and Morris (30) by means of definitive chemical reactions.

Acetoglycerides in Other Oils

Seed oils absorbing strongly in the infrared at 8.1 μ, indicative of the acetate group, are not uncommon. Since such absorption is not necessarily indicative of acetoglycerides (28), analyses by TLC and GLC are necessary before the presence of monoacetotriglycerides in a seed oil can be established. Such confirmation has been obtained for oils of 13 species in 4 plant families (Table II) in addition to those of Balsaminaceae (7,8). Many more species in other plant families show evidence by GLC of small amounts of these triglycerides.

ACKNOWLEDGMENTS

R. G. Powell performed ORD analysis; L. W. Tjarks, NMR spectra; Q. Jones, Crops Research Div., USDA, Beltsville, Md., provided seeds; and W. S. Singleton, Southern Regional Research Laboratory, ARS, USDA, New Orleans, La., supplied purified egg lecithin.

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Fractionation and Analysis of Rat Liver $^{14}CH_3$-Lecithins Labeled in Vivo

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ABSTRACT

The $^{14}CH_3$-lecithins were biosynthesized by normal adult rats injected with $^{14}CH_3$-methionine. About 20% of the dose was incorporated into liver lecithins. The $^{14}CH_3$-lecithins were isolated by thin-layer chromatography. Separation of lecithins on $AgNO_3$-treated silica gel yielded lecithins containing a saturated fatty acid in combination with mainly one unsaturated fatty acid, namely, oleic, linoleic, eicosatrienoic, or arachidonic acid. These fractions were eluted with methanolic choline chloride, which prevented elution of $AgNO_3$. The lecithins, after extraction into petroleum ether, were analyzed for radioactivity and for fatty acid composition. Yields were about 75%, based upon fatty acids or radioactivity applied to the plate.

Specific activities differed sharply between the fractions, and arachidonoyl-lecithins had the highest specific activity. The sum of the activities contributed by each of the fractions agreed well with the specific activity of total lecithins, indicating the recovery of intact lecithin molecules. The recovery of intact molecules allows this procedure to be used with lecithins containing any isotopic labels.

The high specific activity of arachidonoyl-lecithins relative to the other fractions indicates a high degree of specificity in the metabolic reactions which lead to the formation of rat liver lecithins.

INTRODUCTION

RAT LIVER LECITHINS exhibit metabolic heterogeneity when ^{32}P or $^{14}CH_3$-methionine are incorporated in vivo. The methyl label concentrates in lecithin fractions which are rich in arachidonic acid (1,2) whereas the phosphate label is heaviest in fractions with high proportions of palmitic and linoleic acids (1,3,4). This heterogeneity clearly shows that it will be necessary to learn the behavior of individual molecular species of lecithins for a full understanding of lecithin metabolism.

The distinguishing characteristic of any individual lecithin molecule is its fatty acid pair, which usually consists of one saturated fatty acid in the 1-position and an unsaturated fatty acid in the 2-position. Much has been learned about fatty acid-pairing in lecithins from the work of Renkonen (5), Wurster and Copenhaver (6), and Van Golde et al. (7,8). In these studies, fatty acid pairing was determined in lecithins from which the choline group had been removed to facilitate separations. Such procedures, although elegant, could not serve to determine the influence of fatty acid composition on the incorporation of the methyl label, which would require the analysis of intact lecithin molecules.

Previous studies suggested that arachidonoyl-lecithins incorporated most of the activity from $^{14}CH_3$-methionine, but specific activities could not be assigned definitely to any particular unsaturated fatty acid because it was not possible to separate lecithins into distinct fractions (1,2). In 1965 Arvidson published a procedure for separating lecithins according to the degree of unsaturation by thin-layer chromatography (TLC) on $AgNO_3$-treated silica gel (9). This method, if it could be adapted to allow radioactivity measurements, would be ideal for determining the influence of unsaturated fatty acid composition on the incorporation of $^{14}CH_3$-methionine into rat liver lecithins.

Therefore Arvidson's procedure (9) was modified to permit specific activity determinations on lecithin fractions, in addition to the fatty acid-pairing patterns already obtainable. The first efforts to measure radioactivity of methanol eluates, either in scintillation mixtures or on planchets, led to low and erratic recoveries of activity. These also occurred when powder from the TLC plates was counted directly in scintillation mixtures. The presence of $AgNO_3$ was responsible for these results. In the procedure presented below, this difficulty is avoided by elution of lecithins with methanolic choline chloride, which prevents the elution of Ag^+. When this eluate is saturated with choline by the evaporation of methanol, the solubility of lecithins is reduced so that they can be extracted into petroleum ether, which is

easy to remove for the determination of composition and specific activity. Lecithin fractions can be prepared in yields of 75% or more, based on recoveries of fatty acids and radioactivity.

EXPERIMENTAL

Biosynthesis of $^{14}CH_3$-Lecithins

Unfasted, adult male and female Long-Evans rats, which had been maintained on an adequate, semipurified diet (10) for three weeks, were injected intraperitoneally with a saline solution containing 3.3 microcuries of $^{14}CH_3$-methionine (Calbiochem, Los Angeles, Calif.) per 100 g of body weight. Twenty minutes to five hours after injection the animals were killed by decapitation, and the livers were removed and lyophilized. Under these conditions about 20% of the radioactive dose was incorporated into liver lecithins. Analysis of the $^{14}CH_3$-methionine by TLC (95% ethanol-water, 81:37 v/v) showed that at least 98% of the activity migrated with methionine; no other ninhydrin-positive components were present.

Radioactivity Measurements

Radioactivity measurements were made with a Packard Model 314-DC scintillation counter (Packard Instrument Company, LaGrange, Ill.). Theoretical efficiency, as measured with the Packard $^{14}CH_3$-toluene sealed standard, was 54%. Actual efficiency, measured with $^{14}CH_3$-toluene in 0.4% BBOT (in Merck reagent toluene), was 43%. Quenching was checked by use of $^{14}CH_3$-toluene of known dpm. Some samples were scraped directly from TLC plates into a counting solution which contained 0.4% PPO, 0.03% POPOP, and 10% naphthalene (Matheson #2616) in dioxane (Matheson spectroquality reagent), diluted just before use with 0.2 volume of water. The efficiency of the dioxane mixture was about half that of the toluene mixture (BBOT, PPO, and POPOP, Packard Instrument Company).

Analysis of Fatty Acids

Fatty acids were analyzed with Aerograph Model 200 chromatographs (Wilkens Instrument and Research Company, Walnut Creek, Calif.), equipped with hydrogen-flame detectors and silanized DEGS columns. Each instrument was calibrated daily with a standard containing accurately known proportions of the methyl esters of lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidonic acids (Calbiochem, Los Angeles, Calif.; Hormel Institute, Austin, Minn.; Applied Science Laboratories, State College, Pa.). Proportions of fatty acids were determined from the product of the peak height and retention time. The response of methyl palmitate was defined as 100% for the calibration standard, and other components responded within $\pm$ 2% of this value. For example, if methyl palmitate gave a value of (height x time/wt methyl palmitate) = 100, other components gave values between 98 and 102. Corrections were applied for differences in response. The weights of individual components were determined by the use of an internal standard (11).

Methyl esters were prepared by boiling 0.1 to 1.0 mg of lecithin in methanol acidified with H_2SO_4. Heating continued for at least 30 min, until the concentration of acid reached about 10% by volume, total volume 0.2 to 0.5 ml. One-half to two volumes of water were added, and methyl esters were extracted from the aqueous phase into petroleum ether (reagent, distilled over sodium, B.R. 30-60C). Transmethylation was complete in 30 min or less. Extraction tests with the accurately known GLC standard showed that this extraction procedure gave uniform yields of all methyl esters.

Thin-Layer Chromatography

Silica gel H (Merck, Darmstadt) was used for all separations. It was washed with chloroform-methanol-formic acid mixtures, all reagent grade (12). The damp powder was dried at 110C, then pulverized, and sieved through 60 mesh. This purification reduced the discoloration of $AgNO_3$-treated plates and lowered fatty acid values in blanks. DCF (2′, 7′-dichlorofluorescein, Eastman Organic Chemicals, Rochester, N. Y., 0.4% in methanol) and Rhodamine 6G (Allied Chemical Corporation, New York, 0.25% in redistilled 95% ethanol) were used to stain ordinary and $AgNO_3$-treated plates respectively. Rhodamine 6G produced brighter patterns on $AgNO_3$-treated plates than either DCF or 4′,5′-dibromofluorescein (Eastman Organic Chemicals).

Isolation and Characterization of $^{14}CH_3$-Lecithins

Lyophilized livers were pulverized and extracted twice with 100 ml of chloroform-methanol, 2:1, v/v (Merck). Solvents were removed with a rotary vacuum evaporator, and the lipid was taken up into petroleum ether for storage at −20C until analysis. Extracts were pooled to provide a sufficient supply of material for developing the procedure.

Lecithins were isolated from other lipids by TLC, according to Skipski et al. (13). Direct counting of powder scraped from TLC plates into the dioxane-counting mixture showed that 95% of the radioactivity was located in the lecithin band, and the rest was chiefly lysolecithin and sphingomyelin. Lecithin was identified by TLC comparison with authentic lecithin (Sigma Chemical Company, St. Louis, Mo.), with Dragendorf reagent, with ninhydrin (no reaction occurred), and by measurement of the fatty acid:phosphorus (14) mole ratio, which was 2. After acid hydrolysis, choline was the only base detectable by TLC (15) and contained at least 96% of the radioactivity present in the hydrolysate.

Lecithins were prepared on 0.5-mm TLC plates with the solvent system of Skipski et al. (13) (chloroform-methanol-acetic acid-water, 25:15:4:2). About 700 μg of lipid phosphorus were applied in an 18-cm streak. Lecithins were located with DCF. The band was scraped into a 12- or 15-ml centrifuge tube, and the powder was eluted twice with a total of 15-20 ml of 5% choline chloride (Eastman Organic Chemicals) w/v in glass-distilled methanol. Sharpness of separation was confirmed by treatment of the remainder of the plate with iodine vapor. The choline chloride-lecithin solution was reduced 90% in volume under vacuum, and solid choline chloride was added, if necessary, to saturate the solution. This mixture was extracted twice with 5 to 10 volumes of petroleum ether. Eighty per cent or more of the applied lecithin activity was recoverable under these conditions. Lecithins thus prepared were either analyzed immediately or transferred to methanol for storage since the unsaturated fatty acid content decreased if isolated lecithins were kept in petroleum ether for a few days, even at –20C. Lecithin stored in methanol at 4C maintained its fatty acid composition and specific activity for at least two weeks. Rechromatography of isolated lecithin showed that it was free from other lipids and was pure except for a very small component near the origin visible in iodine vapor but not with DCF.

Fractionation of Lecithins According to Degree of Unsaturation

Air-dried silica gel layers, 0.5 mm thick and 20 cm square, were sprayed with 15 ml of 20% aqueous $AgNO_3$, w/v. Plates were air-dried, then baked 15 to 24 hr at 175-180C. About 10 mg of lecithin, in petroleum ether or chloroform, were applied in an 18-cm streak. The plate was chromatographed in chloroform-methanol-water, 65:25:4,v/v (9) in a tank without paper liners. Bands were located with Rhodamine 6G, then were scraped and eluted exactly as described for the isolation of lecithins. Choline chloride precipitated the Ag^+ as chloride and reduced the solubility of lecithin in methanol so that the lecithin could be extracted into petroleum ether. Blank bands were similarly eluted and analyzed, and lecithin samples were corrected for small extraneous GLC peaks found in blanks. Yields were about 75% or more, based on radioactivity or fatty acid recovery.

RESULTS

Fatty acid compositions and specific activities of the rat liver total lecithins are shown in Table I. Saturated fatty acids accounted for about half of the fatty acids in total lecithins and in the diene, triene, and tetraene fractions. Since most of the lecithin molecules contain one saturated and one unsaturated fatty

TABLE I

Fatty Acids and Specific Activities in $^{14}CH_3$-Lecithins of Rat Liver

Fatty acid[1]	Mole percentages of Fatty Acids				
	Total lecithins	Fractions			
		Monoene	Diene	Triene[2]	Tetraene
16:0	24.1	37.8	24.5	17.4	18.4
16:1	2.0	5.3	2.3	1.6	0.6
18:0	28.2	19.6	23.8	33.9	33.7
18:1	10.6	36.1	5.0	4.6	4.2
18:2	11.2	1.2	44.4	8.1	1.2
20:3[b]	3.9	0.0	0.0	34.0	3.7
20:4	20.0	0.0	0.0	0.4	38.2
Specific activity cpm/micromole fatty acid	5243	2862	3810	2465	6927

[1]Number of carbon atoms: number of double bonds.

[2]Sum of two isomers.

TABLE II

Contributions from Each Fraction to the Specific Activity of Total $^{14}CH_3$-Lecithins

Fraction	Mole fraction of total unsaturated fatty acids	cpm/micromole fatty acid	cpm/micromole/fraction
Monoene	0.264	2682	708
Diene	0.235	3810	895
Triene	0.082	2465	202
Tetraene	0.419	6927	2902
Sum	1.00	cpm/micromole calculated	4707
		observed	5243
		Calc./obs. = 89.8%	

acid, the proportion of the total lecithin contributed by each fraction can be approximated from the total lecithin figures. For example, 40 mole % of total lecithins are tetraenes (2 x 20 mole %); monoenes, dienes, and trienes account for about 25%, 22%, and 8% respectively. The sum of these (95%) is less than 100% because of a few of the lecithins contain two saturated fatty acids; these appear in the monoene fraction, which does not separate from fully saturated lecithins (9). The monoene fraction contains 42.6 mole % unsaturated fatty acids so that this fraction has about 85 mole % lecithins with one unsaturated fatty acid. The other 15 mole % is fully saturated. Fully saturated lecithins therefore amount to about 15% x 25% = 4% of total lecithins in agreement with the calculation above.

The purities of the polyunsaturated fractions can be estimated in the same way. The diene fraction contains 44.4 mole % linoleic acid, therefore the fraction is about 89 mole % linoleoyl-lecithins. Similarly triene- and tetraene-lecithins are approximately 68% and 76% pure respectively. In every case there is a small dilution of the fraction with fewer unsaturated fatty acids, which probably resulted from chromatographic trailing although diunsaturated lecithins have been detected in rat liver (7).

A very small triene fraction contained fatty acids identified by their GLC behavior as eicosatrienes, probably the ω-6 and ω-9 isomers, in roughly equal proportions. Hexaenes were negligible, probably because the dietary cottonseed oil furnished very little linolenic acid (ω-3) as its precursor.

The sharp differences between the specific activities of the different fractions show that exchange of fatty acids between different lecithin molecules did not occur since that would have caused uniform specific activities in all fractions.

Table II shows the calculated contribution from each fraction to the total specific activity. The proportion (mole fraction) of each fraction times its specific activity gives the "specific activity fraction." If the lecithin molecules are recovered intact, the sum of the "specific activity fractions" should be the same as the specific activity of the total lecithins. The sum of the activity fractions is actually 89.8% of the measured specific activity of total lecithins, indicating good recovery of intact lecithin molecules. The recovery calculated in this way is less than quantitative, partly because no correction has been made for the dilution of the fractions by other unsaturated fatty acids. For example, the large tetraene fraction is known to be diluted with trienes and dienes, which have lower specific activities (Table I). Therefore the measured specific activity of the tetraene fraction is somewhat lower than it would have been if absolutely pure. The same is true of the diene fraction. The real contributions of these fractions to the total specific activity is therefore larger than the calculation shows so that 89.8% should be regarded as a minimum "recovery" of specific activity. The measured specific activity of the triene fraction is probably higher than the true value for the reason explained above, but this is such a small fraction that an uncertainty in its specific activity affects the calculation very little.

DISCUSSION

The procedure described above serves the main purpose, namely, the determination of specific activity of $^{14}CH_3$-lecithins in relation to their degree of unsaturation. The data also confirm previous suggestions (1,2) that the arachidonoyl-lecithins have the highest specific activities.

This procedure for preparation and analysis of radioactive lecithins with mainly one kind

of unsaturated fatty acid should be usable with any isotopic labels since the molecules are preserved intact. These lecithins could also serve as a convenient starting-point for the separation of such binary mixtures into individual molecular species by reversed-phase TLC, as recently reported by Arvidson (16). This latter procedure appears to be adaptable for radioactive measurements although no specific activity data were presented.

Finally a preparation of naturally-occurring lecithins of relatively simple fatty acid composition should be useful for studies of its physical behavior or interactions with other lipids. At present, such studies are complicated by the presence of many molecular species. Eventually the various functions ascribed to "lecithin" may be correctly assigned to the appropriate structures.

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The Metabolism of 1-^{14}C Arachidonic Acid in Pyridoxine-Deficient and Pair-Fed Control Rats

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ABSTRACT

The metabolism of 1-^{14}C arachidonate was studied in pyridoxine-deficient and pair-fed pyridoxine-supplemented control rats. The studies included intestinal absorption, oxidation to $^{14}CO_2$, organ uptakes and distribution of ^{14}C in the fatty acids of the various organs. Generally, pyridoxine deficiency resulted in little or no alteration of the metabolism of arachidonic acid 6 and 12 hrs after oral administration. A notable exception occurred in hearts of deficient animals in which the proportion of the incorporated ^{14}C activity found in fatty acids other than 20:4 was larger than that observed in hearts of pyridoxine-supplemented animals. A significant amount of ^{14}C activity in a water-soluble form was observed in hydrolysates of intestinal contents and of intestines of both groups. Pyridoxine-deficient rats had larger quantities than their respective pair-fed pyridoxine-supplemented controls. Most of the ^{14}C activity of fatty acids of various organs was present as arachidonic acid, but significant activity was present both in fatty acids of shorter and of longer retention time than 20:4. In brain about 20% of the ^{14}C activity in fatty acids was in a fraction tentatively identified as an 18:2 isomer. In lungs about 10% of the ^{14}C activity was in a fraction tentatively identified as a 22:2 isomer and a similar quantity was observed in a polyene tentatively identified as a 22:4.

INTRODUCTION

THE ROLE OF PYRIDOXINE in fat metabolism has been a subject of interest for many years (1). It received great impetus when Witten and Holman (2) reported that the vitamin might play a role in essential fatty acid conversions in the rat. Past investigations have been concerned primarily with the role of pyridoxine in the conversion of fatty acids to longer chain and more highly unsaturated derivatives. Synthesis and tissue concentrations of arachidonic acid have been principal objectives of such studies. If pyridoxine deficiency actually alters lipid metabolism, it is possible that the alterations may be in the further metabolism of arachidonic acid rather than in its synthesis. Changes in oxidation to carbon dioxide or conversion of arachidonic acid to other metabolites would result in changes in concentration of this essential compound in tissues whether or not conversion of linoleic to arachidonic acid had changed with pyridoxine deficiency. It was the purpose of this investigation to compare the metabolism of orally administered ^{14}C-arachidonate in rats maintained on a pyridoxine-deficient diet with that occurring in rats pair-fed the same diet but receiving an adequate daily dose of pyridoxine.

EXPERIMENTAL

Weanling, male Sprague-Dawley rats were fed a diet free of pyridoxine and containing 20% vegetable shortening[3] for 3 months as described previously (3). Controls were given the same diet plus 20 μg pyridoxine per day and were pair-fed to the experimental. At the end of the 3-month feeding period, a tracer dose containing 3 μc and 2 mg methyl 1-^{14}C-arachidonate in 0.5 ml olive oil was given to the animals by stomach tube and the animals killed 6 or 12 hrs later. The labeled arachidonate (kindly donated by Hoffmann-LaRoche, Nutley, New Jersey) was about 94% pure by gas-liquid chromatography (GLC) and by gas-liquid radiochromatography. Practically all the activity not in the arachidonate fraction was in a fraction having a retention time equivalent to that of 20:3. Because of the small amount of labeled material available no attempt was made to purify the ^{14}C-arachidonate.

After administration of the labeled fatty acid the rats were placed individually in metabolic cages designed for collection of expired air. The carbon dioxide was trapped in sodium hydroxide solution and aliquots taken for

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[3] Crisco, containing added polyunsaturates, Procter & Gamble Company, Cincinnati, Ohio.

determination of total amount of CO_2 expired and for ^{14}C determination. The latter was done by liquid scintillation counting of Ba $^{14}CO_3$ using a thixotropic gel.[4]

Livers were extracted by use of the Folch mixture. The extracts were subsequently evaporated to dryness in a rotary evaporator at room temperature and the residue extracted with a mixture of 90% petroleum ether, 10% chloroform. Aliquots of these were used for determination of radioactivity by liquid scintillation counting, for total fatty acid determination following hydrolysis and extraction, and for separation of various lipid classes using the silicic acid column of Hirsch and Ahrens (4). The purity of the fractions was checked by thin-layer chromatography (TLC).

One testis from each animal was hydrolyzed under nitrogen in 10% ethanolic KOH and the fatty acids resulting after acidification of the hydrolysate extracted with petroleum ether. The other testis of each rat was pooled with others from the same group and used for fractionation of lipid classes. These were homogenized in Folch mixture and treated in a manner similar to the liver.

Analyses of total fatty acids of other organs and tissues were done in a manner similar to the testis.

Methyl esters of fatty acids were prepared using the method of Metcalfe and Schmitz (5). GLC of the methyl esters and gas-liquid radiochromatography of some samples were done as described previously (6). Gas-liquid radiochromatography of samples analyzed in the later stages of the experiment was done by collection of emerging peaks on siliconized glass wool. The glass wool was then transferred to counting vials, and 10 ml of toluene containing diphenyl oxazole and 1,4-bis-2-(5 phenyl-oxazolyl)-benzene added. This mode of collection of methyl esters is similar to that reported by Bennett and Coon (7). Hydrogenation of fatty acids was accomplished using platinum oxide catalyst according to the method of Farquhar et al. (8).

Identity of water-soluble radioactive compounds was attempted by paper chromatography of dinitrophenylhydrazine derivatives with the solvent system n-butanol:ethanol:0.5 N NH_4OH (7:1:2) (9). Standard acetoacetate dinitrophenylhydrazine derivative was prepared from ethyl acetoacetate by the method of Clift and Cook (10).

Serum glutamic-oxalacetic transaminase was determined using Dermatube-SGO[5] which contains the reagent concentrations proposed by Amador and Wacker (11).

RESULTS

The administered ^{14}C-arachidonate was absorbed well (90% to 96% of administered dose) by both groups. About 10% of the oral dose was oxidized to $^{14}CO_2$ in 6 hr and 15% in 12 hr by both groups. In only 2 cases (out of 7) was there slightly more ^{14}C activity expired by pyridoxine-deficient animals than by pyridoxine-fed controls.

The radioactivity of hydrolyzed intestinal contents was present in petroleum ether-soluble and water-soluble forms. The latter constituted 17.3 ± 4.2% of the total ^{14}C counts in the 6-hr pyridoxine-deficient vs. 12.4 ± 4.1 for 6-hr pyridoxine-fed rats. In the 12-hr group the corresponding values were 16.5 ± 5.9 (pyridoxine-deficient) and 4.9 ± 1.1 (pyridoxine-fed). Statistically, these differences were of borderline significance (P=0.1) because of small numbers of samples and large variation in animals of the same group. However, in 4 or 5 pairs of 6-hr samples and in all 3 pairs of 12-hr samples the value obtained for pyridoxine-deficient rat was greater than that obtained for the pair-fed pyridoxine-supplemented control. At least a portion of this water-soluble radioactivity was associated with material which by paper chromatography had an R_f equivalent to that of acetoacetate. Further quantitative studies or chemical identification were not done. Water layers from hydrolysates of intestinal tissue were also checked for ^{14}C activity and results were similar to those obtained for intestinal contents, but the actual values were smaller (4.6% for deficient vs. 1.9% for supplemented).

The ^{14}C content of fatty acids of selected

TABLE I

^{14}C Content of Selected Organs of Rats 12 hr After Oral Administration of 1-^{14}C-Arachidonate

Organ	Percentage of absorbed dose
Intestine	10.4 ± 0.8[a]
Liver	26.3 ± 3.1
Testis	1.4 ± 0.2
Lung	0.60 ± 0.08
Brain	0.15 ± 0.02
Kidney	1.1 ± 0.1
Heart	0.4 ± 0.04

[a] Average ± standard error.

[4] Cab-O-Sil (trademark of Godfrey L. Cabot, Inc.), obtained from Packard Instrument Company, LaGrange, Illinois.

[5] Worthington Biochemical Corporation, Freehold, New Jersey.

TABLE II

Composition of Intestinal Fatty Acids

	Percentage of Total Fatty Acids		
	Pyridoxine-deficient	Pyridoxine-fed	P value
14:0	0.7 ± 0.1[a]	1.1 ± 0.1	<0.001
16:0	18.1 ± 1.1	20.5 ± 0.6	0.04
16:1	1.9 ± 0.2	3.4 ± 0.8	<0.01
18:0	10.4 ± 0.7	6.1 ± 0.8	<0.001
18:1	41.2 ± 2.7	46.3 ± 1.1	0.05
18:2	19.3 ± 0.8	20.1 ± 0.3	0.30
20:4	7.3 ± 1.3	2.5 ± 0.3	<0.001

[a] Average ± standard error.

TABLE III

Distribution of ^{14}C in Fatty Acids of Hearts of Rats Killed 6 hr After ^{14}C-Arachidonate Feeding

Fatty acid fraction	Percentage of ^{14}C counts collected		
	Pyridoxine-deficient	Pyridoxine-fed	P value
<20:3	13.1 ± 2.0[a]	4.1 ± 0.4	<0.01
20:3 } 20:4 }	67.9 ± 2.2	84.4 ± 1.7	0.02
>20:4	19.6 ± 1.3	10.0 ± 1.7	0.01

[a] Average of 3 rats ± standard error.

organs 12 hr after oral administration of the ^{14}C-arachidonate is shown in Table I. Only in kidney was there a difference between pyridoxine-supplemented and pyridoxine-deficient rats (1.1% vs. 1.5% of absorbed dose; $P=0.005$).

The composition of intestinal fatty acids of pyridoxine-deficient differed from that of pyridoxine-supplemented rats. Results of GLC analyses are shown in Table II. Intestines of pyridoxine-deficient animals had relatively more 18:0 and 20:4 than supplemented animals and relatively less 14:0, 16:1 and 18:1.

Differences in fatty acid composition between the two groups were also seen in heart tissue (18:0, pyridoxine-deficient 23.3 ± 1.4, pyridoxine-supplemented 18.7 ± 0.9, $P = 0.03$; 18:1, deficient 20.3 ± 1.0, supplemented 25.8 ± 1.2, $P = 0.01$).

In carcass, serum and most of the organs examined, more than 85% of the ^{14}C activity was in the 20:3, 20:4 fraction as isolated by gas chromatography. However, in heart, lungs and brains considerable quantities of ^{14}C were present in fatty acids having retention times different than the 20:3, 20:4 fractions. Results of gas-liquid radiochromatographic determination from each of 3 hearts from the 6-hr. pyridoxine-deficient and pyridoxine-fed animals are shown in Table III. The pyridoxine-deficient animals had less ^{14}C activity in the arachidonate fraction and a greater proportion in compounds having retention times shorter and longer than 20:4. The labeled compounds having shorter retention times than 20:4 were predominantly those having retention times equivalent to 16:0, 18:0 and 20:2. Those having retention times longer than 20:4 were compounds having retention times equivalent to those of 22:3 and 22:4. Further identification of these could not be done because of unavailability of material.

Studies of ^{14}C distribution in fatty acids of lung tissue revealed significant amounts of ^{14}C activity (10% of total) in a fraction having a retention time equivalent to that of 22:2 and similar amounts in one having a retention time equivalent to that of 22:4. Pooled samples of lung fatty acids were used for isolation of pure 22:2 and 22:4 in larger quantities by GLC. After purity of the isolated 22:2 was established by gas-liquid radiochromatography, a portion was hydrogenated. The hydrogenated sample was chromatographed and the effluents collected in cartridges packed with silicone-coated glass wool. The only ^{14}C activity detected was in the fraction emerging with a retention time of 22:0, confirming the identity of the material isolated originally as a 22-carbon unsaturated fatty acid. Similar procedures were used to establish the identity of the compound emerging with a retention time of 22:4.

In brain tissue from both groups of animals about 40% of the ^{14}C was in fatty acids other than 20:4. Significant amounts of radioactivity were found in 16:0, 18:0 and 18:1 but about half of the activity not in the 20:4 peak was in a compound emerging with a retention time of 18:2. This fraction was isolated in larger quantities from pooled brain methyl esters and the purity established by gas-liquid radiochromatography. Part of the isolated fraction was rechromatographed and only ^{14}C activity emerging from the gas chromatograph had a retention time of 18:2. Another portion was hydrogenated and the hydrogenated sample analyzed by gas-liquid radiochromatography. The ^{14}C activity all emerged with a retention time of 18:0.

Small but significant ^{14}C activity was also observed in compounds of retention time equal to 20:5, 22:4 and 22:6. In the case of testicular tissue significant amounts of ^{14}C activity were observed also in 22:5.

In both pyridoxine-deficient and pyridoxine-fed animals most of the ^{14}C in liver was in the phospholipid fraction (>85%). About 10% was found in glycerides and about 5% in the cholesterol ester fraction. A small amount of activity was observed in free fatty

TABLE IV

^{14}C Distribution in Fatty Acids of Liver Lipids

	Percentage of ^{14}C		
	<20:3	20:3 20:4	>20:4
Phospholipids	4.2	89.6	6.2
Cholesterol esters	3.4	81.2	15.4
Glycerides	8.3	69.7	22.0
Free fatty acids	6.0	86.5	7.5

acids. ^{14}C distribution in fatty acids of the various lipid classes was similar for deficient and supplemented animals. Most of the ^{14}C activity in liver lipids was in the 20:3, 20:4 fractions. However, the cholesterol ester and glyceride fractions had much more activity in compounds of retention time greater than 20:4 than did the phospholipid and free fatty acid fractions (Table IV). The bulk of this activity was in the 20:5 fraction but significant quantities were found also in fractions having retention times identical with those of 22:2, 22:3, 22:4 and 22:6.

In testicular tissue arachidonic acid was the major labeled fatty acid of all lipid fractions 6 hr. after administration of ^{14}C-arachidonate. However, at 12 hr. the cholesterol ester and phospholipid fractions contained ^{14}C activity in larger amounts in fatty acids other than 20:4 (phospholipids: 16:0, 17%; 20:2, 10%; >20:4, 22%).

DISCUSSION

The degree of deficiency may be judged by the difference in body weight between the pyridoxine-deficient and pyridoxine-fed groups. The former averaged 133 g and the latter 220 g after 3 months on the diet. Furthermore, values for serum glutamic-oxalacetic transaminase were about threefold higher in pyridoxine-fed animals compared to the pyridoxine-deficient. A number of the animals on the deficient diet died during the course of the experiment.

The results of these studies show that there is little or no alteration of the metabolism of orally administered ^{14}C-arachidonic acid in pyridoxine deficiency. Our experiments were terminated at 6 and 12 hr. after feeding the ^{14}C compound. Therefore, changes which might become detectable only after several days would be missed in these studies. Experiments of longer time duration are currently underway in our laboratory.

A relatively large amount of activity was found in water-soluble metabolites in hydrolysates of intestinal contents. ^{14}C activity of water-soluble compounds was not determined in other organs in this study, but similar observations were made previously in studying the fate of ^{14}C-arachidonic acid (12) and ^{14}C-linoleic acid (13) in rats fed a fat-free diet. Deficient animals in previous experiments had more ^{14}C counts in such products than the pair-fed controls. In the present experiment the same type of result was obtained although the differences were of borderline statistical significance because of the variation encountered. Tentative identification of part of this material as acetoacetate leads to the interesting concept that the animals "waste" part of their fatty acid fuel in incomplete oxidation. In accord with this concept is the large difference in body weight of the two groups in spite of pair-feeding. Weights of organs of deficient animals were somewhat smaller than those of supplemented animals. Changes in fatty acid composition (Table I) might also reflect a wasting condition. The concept of wasting metabolic fuel in pyridoxine deficiency is in keeping with results reported by Beaton et al. (14) in 1954. Their studies compared rats on a pyridoxine-free diet with control animals receiving the diet plus the vitamin supplement and pair-fed to the deficient. They concluded that the rats not receiving the vitamin were unable to utilize food materials with enough efficiency to take care of their energy requirements and anabolic needs. However, they did not observe any significant differences in fasting blood ketone bodies between the two groups.

In almost all of the tissues examined the ^{14}C activity present in fatty acids was predominantly still 20:4 (i.e., the same as the administered arachidonate). A notable exception was observed in hearts of pyridoxine-deficient rats. In these animals a much greater percentage of the ^{14}C in heart lipids was present in fatty acids other than 20:4 than in heart lipids of pyridoxine-fed controls. Similar observations had been made in rats maintained on fat-free diets and given ^{14}C-arachidonate (12). Apparently, both types of dietary deficiencies stimulate catabolism of labeled arachidonate in heart tissue. The labeled small fragments resulting from this degradation are then utilized for synthesis of fatty acids by de novo or elongation mechanisms. Labeled arachidonate itself may also be elongated to produce labeled compounds with retention times greater than 20:4. Since chemical degradation of the labeled compounds was not done, the mode of

synthesis cannot be specified.

Many organs had significant ^{14}C activity in one or more fatty acids having retention times equal to those for 20:5, 22:2, 22:3, 22:4, 22:5 and 22:6. In the case of fatty acids from lungs and from brain, two of these were shown to be 22:2 and 22:4. In brain tissue about 40% of the ^{14}C activity was in fatty acids other than 20:4 and of this about half was in a fraction tentatively identified as 18:2. Though the chemical identity of this compound was not established further, it may be $\Delta^{8,11}$ octadecadienoic acid, synthesized from Δ^{9} 16:1, or $\Delta^{6,9}$ octadecadienoic acid. The presence of the latter isomer in pig brain tissue has been reported by Kishimoto and Radin (15). Formation of $\Delta^{6,9}$ octadecadienoic acid from ^{14}C-oleic acid by rat liver microsomes was shown by Holloway et al. (16). An 18:2 fatty acid (presumably the same isomer) was shown to be synthesized from 1-^{14}C-acetyl CoA by subcellular particles of rat liver by Harlan and Wakil (17). We have previously also observed biosynthesis of this isomer by rat liver microsomes incubated with ^{14}C-acetyl CoA (18).

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Resistance of Certain Long-Chain Polyunsaturated Fatty Acids of Marine Oils to Pancreatic Lipase Hydrolysis

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ABSTRACT

When whale oil triglycerides were subjected to pancreatic lipase hydrolysis, eicosapentaenoic and docosahexaenoic acids were found mainly in the di- and triglyceride products, suggesting that they are in the 1,3-positions but resistant to the action of the lipase. Their presence in the 1,3-positions was confirmed. Their resistance to pancreatic lipase hydrolysis was demonstrated by analysis of the products of the enzyme action on: (a) a concentrate of highly unsaturated whale oil triglycerides; (b) the latter after randomization; and (c) synthetic 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol.

Docosapentaenoic acid was also shown to be present in the 1,3-position of whale oil triglycerides but was not lipase resistant. It is postulated that the presence of a double bond near the carboxyl group exercises an inhibitory effect, or that the location of the double bonds in the resistant acids places their terminal methyl groups close to the carboxyl, producing a steric hindrance effect.

INTRODUCTION

In a study of the structure of marine mammal oils by the use of pancreatic lipase, the distribution of fatty acids in the hydrolytic products of whale oil suggested that eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids, but not docosapentaenoic (22:5) acid, are resistant to the action of that hydrolytic enzyme. The results of the present study confirm the resistance of those acids to pancreatic lipase action, even though the acids are located in the 1,3-positions of whale oil triglycerides. A preliminary report of this work has been presented (1).

EXPERIMENTAL

The location of the 20:5, 22:5, and 22:6 acids in the whale glyceride molecules and the resistance of these acids to the activity of pancreatic lipase were determined by analyses of the products of the enzyme action on: (a) unmodified whale oil; (b) a concentrate of highly unsaturated whale oil triglycerides; (c) the latter after randomization; and (d) synthetic 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol.

Methods

The triglycerides of two samples of whale oil[1] were purified by preparative thin-layer chromatography (TLC). A highly unsaturated fraction was prepared from one of them by crystallization at –60C (2). Menhaden oil was provided by the Department of Oceanography, Texas A&M University. Lipase (EC 3.1.1.3) from hog pancreas, PL-III, was purchased from Worthington Biochemical Corporation, Freehold, N. J. Lipase hydrolyses were performed in vitro by the procedure of Luddy et al. (3), including the determination of the fatty acid composition of the free fatty acids and of the mono-, di-, and triglyceride products.

Randomization of the highly unsaturated concentrate of whale oil was achieved by treatment with 0.1 M lithium secondary butylate in dimethyl formamide (4). The reaction mixture was kept under nitrogen at room temperature for 3 days. The rearranged triglycerides were purified by preparative TLC.

Purification of triglycerides by TLC was achieved on 0.25-mm thick layers of silica gel (Adsorbosil-1, Applied Science Laboratories, State College, Pa.) on 20 x 20 cm glass plates. The developing solvent system was a mixture of petroleum ether (30-60C bp)-ethyl ether-acetic acid (60:40:1.6, v/v/v).

Gas-liquid chromatography (GLC) was performed in a Research Specialties Model 600 gas chromatograph (Warner-Chilcott Laboratories Division, Richmond, Calif.). The chromatograph was equipped with an argon ionization detector and a 6 ft x 1/4 in. column packed with 15% diethylene glycol succinate on 60-80 mesh Chromosorb W. The column was operated isothermally at 195C. The identities of the quantitatively more important peaks were ascertained by comparing their relative retention times with those of known standards.

[1] One of the samples of whale oil was from the Arista Company, New York. The other was obtained through the courtesy of H. S. Olcott.

Infrared spectra were obtained in a IR8 Beckman infrared spectrophotometer between sodium chloride pellets.

1,2-Di-octadecenoyl-3-eicosapentaenoyl glycerol was synthesized from 1,2-diolein and eicosapentaenoyl chloride and purified by TLC. A manuscript describing this synthesis is in preparation. Eicosapentaenoic acid, 91% pure, isolated from menhaden oil, was purchased from the Hormel Institute, Austin, Minn.

RESULTS AND DISCUSSION

Evidences of Resistance

After 50% pancreatic lipase hydrolysis of the whale oil triglycerides, the concentrations of the 20:5 and 22:6 acids were lower in both the fatty acid and the monoglyceride fractions, but higher in the diglyceride and triglyceride fractions of the resultant mixture than in the original oil (Table I). This suggests that these two polyunsaturated fatty acids are in the 1- and 3- positions but are resistant to the action of the lipase. That the 20:5, 22:5, and 22:6 acids of the whale oil are in the 1,3-positions has been reported by Brockerhoff and Hoyle (5). The accumulation of long-chain polyunsaturated fatty acids in the diglycerides after lipase hydrolysis of marine oils has also been reported by others (4, 6, 7).

Not all the polyunsaturated acids of whale oil behave as the 20:5 and 22:6 acids. The 22:5 acid was present in the free fatty acids and was not enriched in the di- and triglycerides, although like the 20:5 and 22:6 acids it was in low concentration in the monoglyceride products of hydrolysis (Table I). Therefore, the 22:5 acid must be considered as also present in the 1,3-positions; but, in contrast to the 20:5 and 22:6 acids, susceptible to the action of pancreatic lipase.

TABLE I

Major Fatty Acid Components of Whale Oil Triglycerides and Its Lipase Hydrolysis Products

Acid[a]	Sample	Whale oils				
		Original TG	Products of hydrolysis[b]			
			FA	MG	DG	TG
		percentage				
14:0	I[c]	8.7	8.1	9.3	9.2	7.1
	II	4.6	3.4	7.7	3.9	2.4
16:0	I	14.8	20.1	5.5	9.9	12.5
	II	14.9	21.1	10.0	7.9	7.3
16:1	I	16.7	11.3	28.9	17.8	12.0
	II	14.4	9.2	24.1	16.6	8.6
18:1	I	32.2	37.5	40.9	29.4	23.7
	II	33.6	38.7	45.0	30.1	19.0
20:1	I	2.6	5.3	0.3	1.1	1.9
	II	2.1	2.3	1.1	2.1	2.8
20:5	I	6.6	2.0	2.1	11.8	13.8
	II	8.1	3.0	2.4	17.7	26.0
22:5	I	3.9	2.9	0.5	2.2	2.9
	II	5.2	5.9	tr	3.9	6.8
22:6	I	4.9	2.6	0.8	5.3	10.1
	II	5.8	3.9	tr	8.1	17.3

[a] Chain length: number of double bonds.

[b] FA = Fatty Acids; M = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

[c] Average of duplicate analyses.

Since the concentration of some of the polyunsaturated acids were low in the original whale oil, a highly unsaturated concentrate was obtained by removal of the more saturated glycerides by crystallization from acetone at –60C (2). The concentrate was then subjected to pancreatic lipase hydrolysis. The results are presented in Table II-A. It can be seen that, as compared to a level of about 22% in the concentrate, there were only 7% and 8% of the 20:5 acid in the free fatty acid and monoglyceride fractions, respectively. There

TABLE II

The Effect of Randomization on the Products of Pancreatic Lipase Action on a Highly Unsaturated Fraction from Whale Oil Triglycerides (major fatty acids only)

Acid[b]	(A) Whale oil highly unsaturated TG[a]					(B) Randomized whale oil highly unsaturated TG					(C) Recalculation of (B) omitting 20:5 and 22:6				
	Concentrate (original TG)	Products of hydrolysis				Randomized TG	Products of hydrolysis				Randomized TG	Products of hydrolysis			
		FA	MG	DG	TG		FA	MG	DG	TG		FA	MG	DG	TG
	percentage					percentage					percentage				
14:0	4.9	4.1	6.3	4.1	4.7	4.6	8.5	7.3	4.6	4.3	6.9	9.0	9.5	7.7	7.6
16:0	2.4	4.6	1.1	1.3	2.4	2.3	5.1	3.0	2.0	2.0	3.5	5.4	3.9	3.4	3.5
16:1	15.6	16.9	34.5	20.3	12.1	14.1	24.0	18.6	14.6	12.9	21.3	25.5	24.2	24.5	22.8
18:1	25.5	38.0	27.0	20.0	19.4	25.3	35.1	26.8	17.1	17.8	38.2	37.3	34.9	28.7	31.5
20:1	3.5	2.3	3.2	3.6	3.1	3.1	1.5	2.7	4.5	3.9	4.7	1.6	3.5	7.6	6.9
20:5	22.3	7.0	8.2	26.4	30.1	22.4	3.2	16.4	29.7	29.6					
22:5	4.6	8.0	0.9	3.1	4.3	4.8	3.6	4.5	5.2	5.1	7.2	3.8	5.9	8.7	9.0
22:6	12.6	8.5	2.0	11.4	15.9	11.3	2.8	6.8	10.8	13.9					

[a] FA = Fatty Acids; MG = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

[b] Chain length: number of double bonds.

TABLE III

Major Fatty Acid Components of Menhaden Oil Triglycerides and Its Lipase Hydrolysis Products

Acid[a]	Original TG	Products of hydrolysis[b]			
		FA	MG	DG	TG
	percentage				
14:0	11.1	11.0	14.2	7.7	6.3
16:0	19.4	27.2	24.9	14.7	17.4
16:1	16.1	17.6	13.6	9.1	8.1
18:0	5.6	7.6	3.2	3.4	3.7
18:1	16.2	20.1	5.7	4.7	5.0
20:1	3.8	1.6	2.8	6.6	6.0
20:5	10.5	2.0	11.4	22.8	25.5
22.5	1.4	0.6	2.5	2.2	tr
22:6	7.3	1.5	15.1	15.0	16.0

[a] Chain length:number of double bonds.

[b] FA = Fatty Acids; MG = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

were 26% in the diglycerides and 30% in the triglycerides. The results from the concentrate thus reinforce previous indications of resistance. The distribution of the 22:6 acid in the hydrolysis products also indicates resistance but to a somewhat lesser degree. The 22:5 acid was hydrolzed normally as shown by its relatively high level in the fatty acid fraction.

In order to rule out position in the triglyceride molecule as the determining factor in the low degree of hydrolysis of the 20:5 and 22:6 acids, an aliquot of the highly unsaturated concentrate was randomized by chemical treatment. Whale oil offers unusual resistance to rearrangement by the use of standard procedures. Several combinations of catalysts, solvents and different times of treatment were tested before satisfactory results could be obtained. Sodium methoxide in methanol solution produced methyl esters difficult to separate from the randomized triglycerides. A xylene suspension of the same catalyst (8) was only partially effective. Lithium secondary butylate in dimethyl formamide solution (4) was found to be effective when the reaction period was prolonged for 3 days at room temperature. This procedure was therefore used. The randomized triglyceride products, purified by TLC, were analyzed by GLC and subjected to pancreatic lipase hydrolysis. The results are presented in Table IIB. Since the fatty acid compositions of the four products of hydrolysis are not similar, one might conclude that the randomization is incomplete. However, this criterion would only be valid if all the acids were equally susceptible to the lipase, a condition which is not met due to the presence of the resistant 20:5 and 22:6 acids. If the data are recalculated omitting the 20:5 and 22:6 acids or, in other words, making the nonresistant acids equal to 100%, the figures shown in Table IIC are obtained. The quite similar concentrations of the six major acids in all four fractions indicates effective randomization.

The presence of significant amounts of 20:5, 22:5, and 22:6 acids in the monoglycerides after, but not before randomization (Table IIB), indicates that they were not originally located in the 2-position in the whale oil triglycerides. Finally, the very low levels of 20:5 and 22:6 acids in the free fatty acid fraction of the pancreatic lipase hydrolysis products of the randomized oil indicate that the reduced degree of hydrolysis of those acids is not due to the positional specificity of the enzyme, but is due to a characteristic of the fatty acid molecule itself.

In order to compare the behavior of the 20:5 and 22:6 acids in the pancreatic lipase hydrolysis of whale oil with their behavior when located mainly in the 2-position as in fish oils, menhaden oil triglycerides were subjected to pancreatic lipase hydrolysis (Table III). The distribution of the 20:5 and 22:6 acids in the hydrolysis products of menhaden oil is different from that in whale oil products (Table I), although their concentrations in the two oils are quite similar. This is further evidence that the distribution of these acids in the two oils is different and that in whale oil hydrolysis their resistance to pancreatic lipase is independent of their position.

It required about 2 min to attain 50% hydrolysis of the untreated whale oils under the conditions used. An extended reaction time should increase the general degree of hydrolysis but leave higher concentrations of the resistant 20:5 and 22:6 acids in the unhydrolyzed di- or triglycerides. This was found to be true only for 20:5, whose concentrations after 2, 3, and 5 min of hydrolysis were 11.8, 14.4 and 18.2% respectively in diglycerides and 13.8, 13.3, and 19.7% respectively in triglycerides. The concentration of 22:6 after 2, 3, and 5 min of hydrolysis was 5.9, 5.3, and 5.4% respectively in diglycerides and 11.0, 7.2, and 6.7% respectively in triglycerides. The lack of increase in percentage of 22:6 in the di- and triglycerides with time might be due to its having approached maximum levels at the 2-min period.

It was also found that the rate of hydrolysis decreased appreciably after half the triglyceride acids were released. This is a logical consequence of distribution in the 1,3-position of the 20:5 and 22:6 acids, their resistance to hydrolysis, and the reported presence of the

C_{29} and C_{22} acids in only 50% of whale oil triglycerides (2).

Proof of Resistance

Proof of the resistance of the 20:5 acid (and by inference of the 22:6 acid) was obtained by study of the action of pancreatic lipase on synthetic 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol. The results are presented in Table IV. The fatty acid compositions of the triglycerides before lipase hydrolysis and of the monoglyceride and triglyceride products of hydrolysis show that the substance synthesized is, in fact, 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol, with some contamination due to impurities in the starting materials.

The experimental values for the composition of the fatty acid and diglyceride fractions are closer to the values calculated on the assumption of resistance than on the assumption of nonresistance. The small amount of monoglycerides produced is another indication of resistance. The presence of 17% 20:5 acid in the fatty acid fraction indicates that some hydrolysis of that acid took place. This could be due to the resistance to the enzyme not being absolute, to the presence of a hydrolyzable isomer of the 20:5 acid in the starting material, or to an alteration in the structure of the all *cis* 20:5 acid during the chemical synthesis of the triglyceride. Analyses of the starting material showed that there were 9% impurities as ascertained by GLC and that only 75% of the theoretical amount of glutaric acid was produced by $KMnO_4$ oxidation in acetic acid medium (9). Examination of the original 20:5 acid and the 1,2-di-octadecenoyl-3-eicosapentaenoyl glycerol by infrared spectrometry showed that only traces of *trans* isomerization occurred during the synthesis.

Mechanism of Resistance

It is evident that in spite of being located at the 1,3-positions of the whale oil triglycerides, the 20:5 and 22:6 acids resist pancreatic lipase hydrolysis while the 22:5 acid is hydrolyzed without difficulty. The explanation for this phenomenon may lie in differences in their molecular structures:

20:5 $CH_3CH_2(CH{=}CHCH_2)_5 - CH_2CH_2COOH$

22:5 $CH_3CH_2(CH{=}CHCH_2)_5 - CH_2CH_2CH_2CH_2COOH$

22:6 $CH_3CH_2(CH{=}CHCH_2)_5 - CH{=}CHCH_2CH_2CO^3OH$

In view of the evidence presented by others (10) the ω3 structure is assumed for these three acids. Since their terminal 17 carbon chains are identical, any differences in behavior must be assumed to be caused by differences in their structure at the carboxyl end of the chain. The responsible factor could be the proximity of the double bond to the carboxyl group, since the first double bond of the resistant 20:5 and 22:6 acids lies closer to the carboxyl group than does that of the nonresistant 22:5 acid. This view is strengthened by the demonstration by Kleiman et al. (11) that the *trans*-3-enoic acids of *Grindelia oxylepis* seed oil are also resistant to lipase hydrolysis. The presence of methyl groups in a position close to the carboxyl end has also been shown to hinder hydrolysis by the lipase (12).

Another difference in structure between the resistant and the susceptible polyunsaturated acids lies in the space relations of their terminal methyl to their carboxyl groups. As shown in the photographs of the molecular models (Figure 1) the terminal methyl groups of the resistant acids lie close to their carboxyl groups. This proximity may cause a steric hindrance effect on the hydrolysis by the lipase.

Metabolic Implications

The resistance of some of the polyunsaturated fatty acids of whale oil to pancreatic lipase hydrolysis provides an explanation for the finding by Garton et al. (13) that whale

TABLE IV

Products of the Action of Pancreatic Lipase on 1,2-Di-octadecenoyl-3-eicosapentaenoyl Glycerol

Acid[b]	Original TG	Products of hydrolysis[a] FA	MG	DG	TG
	Mole percent[c]				
	Theoretical (nonresistance)				
18:1 + impur.[d]	69.7	54.6	100	77.7	69.3
20:5	30.3	45.4	0	22.3	30.3
	Theoretical (absolute resistance)				
18:1 + impur.	69.7	100	[e]	54.5	69.7
20:5	30:3	0	[e]	45.5	30.3
	Experimental				
18:1 + impur.	71.5	83.0	99.1[f]	60.8	73.0
20:5	28.5	17.0	0.9[f]	39.2	27.0

[a] FA = Fatty Acids; MG = Monoglycerides; DG = Diglycerides; TG = Triglycerides.

[b] Chain length: double bond.

[c] The detector response to the 20:5 acid was found to be 0.88 times that of the 18.1. However, no correction was applied since it would have had no significant effect on the conclusions.

[d] The preparation of 20:5 acid used had 8.9% impurities of other fatty acids. Since they are not expected to be lipase resistant, their percentages are added to that of oleic acid.

[e] No MG should be obtained.

[f] Very small amount of MG obtained.

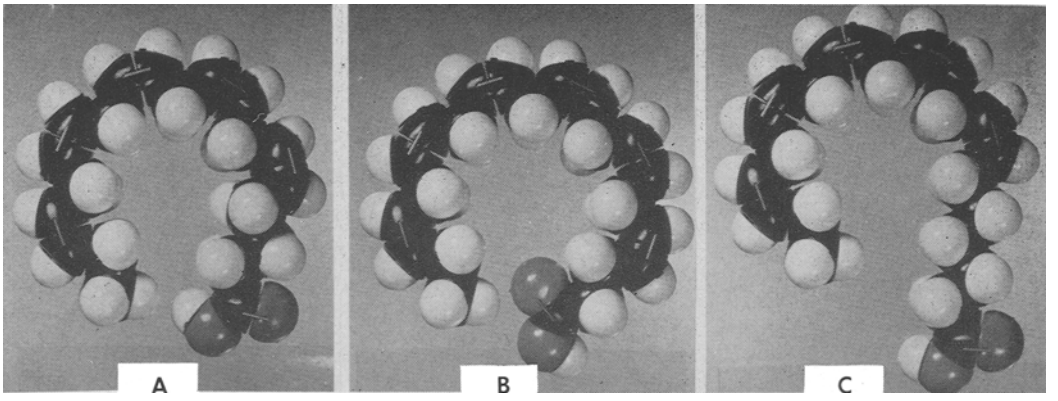

FIG. 1. Molecular models of the 20:5 (A), 22:6 (B), and 22:5 (C) acids of marine oils.

oil can be crystallized almost unchanged from the depot tissues of pigs fed high doses of the oil for a prolonged period of time. In preliminary experiments in this laboratory, however, neither the triglycerides, nor the phospholipids of thoracic duct lymph of rats administered by stomach tube one dose of the highly unsaturated concentrate of whale oil, contained the marine long-chain polyunsaturated acids. The presence of whale glycerides in the tissues of Garton's pigs may have been the product of a low degree of intestinal absorption over a long period of ingestion.

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Brain Cholesterol. XII. The Incorporation of 1-^{14}C-Acetate into Baboon Sterol

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ABSTRACT

The incorporation of 1-^{14}C-Acetate into tissue cholesterol of the baboon was measured. Using this indicator gray matter of the cerebrum indicated greater metabolic activity than did white matter.

Other tissues besides neural tissue were examined. The peak of radioactivity occurred between 3 and 4 hr. The highest incorporation of radioactivity was measured in the adrenal gland. Liver, spleen and kidney values were of intermediate order.

INTRODUCTION

CONTRARY TO EARLIER CONCLUSIONS of others (1), our investigations in the mouse (2-6) indicated that active lipid metabolism continues in the brain during adult life. Because of these preliminary conclusions, and possible relationship to cerebral function, further investigations on brain cholesterol were made. The following is a continuation of the study of cholesterol biosynthesis in primates. The present investigation brings us closer to understanding similar processes in man by reporting on acetate incorporation into brain tissue cholesterol of the baboon.

EXPERIMENTAL

Five male African baboons weighing between 21 and 25 kg., representing ages approximately 4-6 years, were used for these studies. Live animals were imported from Kenya, East Africa, and acclimated for 3 to 4 months before use. Diet, during captivity, consisted of Purina Monkey Chow supplemented with fresh fruit and whole corn.

Anesthetized animals were used in these experiments. Both Nembutol (5 mg/kg) and phencyclidine (Sernylan) (2 mg/kg) were used. The unconscious animals were injected intravenously with 100 μg/kg of 1-^{14}C-Acetate and then killed at five time intervals: 171, 184, 188, 248, and 294 min.

For analysis, samples of cerebrum white, cerebrum gray, liver, spleen, kidney and adrenals were surgically removed, then washed, frozen and stored in an ice chest. Free and esterified sterols (after hydrolysis) were isolated, measured, and assayed by the method previously reported (7). Radiochemical purity of the isolated free cholesterol was established (7,8).

The resulting data are reported in terms of specific activity, (D/M/mg) and activity per gram tissue (D/M/g tissue). Because of uncertainties in the sterol ester measurements, concentration of ester in the individual tissues is not reported at this time.

RESULTS

Values for cholesterol specific activity (D/M/mg), after 1-^{14}C-Acetate injection, are affected by many factors; of those that can be readily ascertained, one is the dilution of specific activity by sterol concentration in each individual organ. To measure the effect of sterol concentration, radioactivity was calculated on a gram tissue basis (D/M/g tissue). Results of both calculations are presented in Table I.

The specific activity of free cholesterol was lowest in cerebrum (white and gray) and highest in the adrenal gland. Liver, spleen, and kidney values were of intermediate order. D/M/g tissue values from these same tissues indicated a similar relative order of incorporation.

The two large divisions of brain matter, white and gray, vary in biosynthetic capacity as measured by 1-^{14}C-Acetate incorporation. By this measure, gray matter of the cerebrum seemed metabolically more active than did the white matter. These differences were evident in comparisons of both free specific activity and activity per gram tissue values.

It should be noted that the specific activity values for ester cholesterol are higher than for free cholesterol. This is true for all tissues under investigation. Because the hydrolysis of the esterified fraction was thought not to be quantitative in these particular experiments, only ester-specific activity values are reported at this time.

Blood values were not available for these studies.

TABLE I

Incorporation of 1-^{14}C-Acetate into Tissue Cholesterol of the Baboon

Organ	Free			Ester
	mg/g Tissue	D/M/mg	D/M/g Tissue	D/M/mg
Cerebrum				
White				
2:51 hr	16.7	27	450	356
3:04 hr	17.2	75	1285	350
3:08 hr	22.0	26	570	429
4:08 hr	13.7	66	905	264
4:54 hr	18.5	40	740	157
Gray				
	11.7	103	1200	2600
	12.7	97	1230	1000
	13.7	55	755	1900
	8.75	308	2695	2238
	13.5	93	1250	1615
Liver	1.35	458	618	15733
	1.04	2148	2234	28944
	0.99	5604	5565	31257
	1.18	1450	1711	76519
	1.34	4291	5750	22116
Spleen	1.13	912	1031	11326
	1.48	3677	5442	8675
	1.07	707	756	6855
	1.73	5551	9603	13465
	1.08	203	219	7326
Kidney	2.00	101	202	55238
	1.27	268	340	22128
	2.08	121	252	50850
	2.06	994	2048	15446
	2.10	115	242	23075
Adrenals	1.84	4713	8672	19743
	1.68	8746	14693	12333
	2.24	3346	7495	11486
	2.27	5065	11498	12297
	2.29	2429	5562	7713

DISCUSSION

More knowledge of the basic biochemical mechanism taking place in neural tissue is needed before we can understand the mechanism of brain function. Studies dealing with the neurochemistry of higher primates, especially man, are extremely scarce. As an approach to this problem, we have investigated the biosynthesis of cerebral cholesterol in the baboon. There are comparatively few recorded studies of the biosynthesis of cholesterol in this primate. Savage et al. (9) reported on the biosynthesis of cholesterol from 2-^{14}C-Sodium pyruvate in normal and diabetic baboons. These workers reported only total radioactivity in various organs at a given time after isotope administration.

Kritchevsky et al. (10,11) studied the biosynthesis of cholesterol with labeled mevalonic acid as the precursor. The peak specific activity of the serum-free cholesterol was attained between 4 and 10 hr; peak specific activity of serum cholesterol ester was reached between 9 and 26 hr. No data on cerebral biosynthesis were presented.

This paper represents our initial research studies on brain colesterol in the baboon. Certain weaknesses of our approach are evident: absence of blood values and the nonquantitative recovery of esterified cholesterol. Despite these shortcomings, the data proved of value as a basis for future studies.

Because of the difficulty in obtaining large primates and their concurrent cost, only 5 animals were used for this experiment; data from these animals did not indicate peak time of labeling. It was expected that in such a small series, individual biological variations might be large. In collecting the data, it was noted that the animal killed at 4:08 hr exhibited variance from the other 4 baboons. This was true for all tissue values of this animal. If we exclude this animal from our consideration, a better and probably accurate picture seems to emerge. The exclusion of the single aberrant animal indicates that the peak of tissue labeling took place between 3 and 4 hr.

Of the tissues examined, biosynthetic capacity as measured by 1-^{14}C-acetate incorporation, showed the adrenal gland to be most active, followed by liver, spleen, and kidney. Brain cholesterol metabolism was low but significant in the adult baboon. This agreed with previous findings in mice (2-6) using 1-^{14}C-Acetate. A better indicator of brain synthetic capabilities in regard to sterol synthesis would be U-^{14}C-glucose (5).

Of particular interest and confirming our past results in mice (6) was the high specific activity of cholesterol ester in contrast to free sterol. These ester values were interpreted to mean that equilibration of newly synthesized free cholesterol with total organ cholesterol were slower than the esterification reaction. This may be interpreted as evidence for the existence of at least two free sterol pools.

Of additional interest was the difference in metabolism between cerebral gray and white tissue. In this baboon experiment with 1-^{14}C-Acetate as the precursor, two facts emerge about brain cholesterol metabolism. First, higher activity was measured for cholesterol extracted from gray cerebrum than white cerebrum matter. Second, in contrast to gray cerebrum matter and other tissues, the ester activity of cholesterol assayed from white cerebrum matter was higher on a D/M/g tissue than D/M/mg basis. This is the result of the greater amount (mass) of ester found in white material as compared to gray.

In agreement with our own in vivo findings, were the results reported by Davison and coworkers (12) in rabbits. Utilizing labeled cholesterol, they found the turnover of sterol to be greater in gray cerebrum than in white. In contrast, Korey (13) found that using labeled mevalonate or glucose in vitro, white cerebrum matter (rat) was more active in lipid synthesis than the gray of cerebral cortex.

As measured by present methodology, the problem of relative metabolic activity between gray and white cerebrum matter is still to be solved. Final conclusions concerning brain cholesterol metabolism must wait until more in-depth experiments are made in a variety of species and with different precursors under controlled conditions. More definitive experiments regarding regional cholesterol biosynthesis in the brain of baboons are presently being conducted in this laboratory by the simultaneous use of 2-^{3}H-Acetate, 1-^{14}C-Acetate, or U-^{14}C-glucose.

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Maternal Diet and Brain Fatty Acids in Young Rats

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ABSTRACT

In order to determine to what extent maternal diet influenced the brain lipids of young rats, female rats were maintained on diets differing in fatty acid composition. Fatty acid determinations on the total brain lipids of the young from these dams indicated that the maternal dietary lipids influence the polyunsaturated fatty acid composition of these animals. A maternal diet with a high linoleic-linolenic acid ratio (corn oil) resulted in lower levels of 22:6ω3 and higher levels of 22:5ω6 than one with a low linoleic-linolenic acid ratio (grain). Transfer of young rats at birth to a foster mother, which was fed a diet differing from that of the natural dam, resulted in brain polyunsaturated fatty acid patterns at weaning similar to those of the natural young, and suckling, of the foster mother, thus indicating that the maternal diet in the immediate postnatal period can modify the brain lipids of young rats prior to weaning. The brain lipids of young rats from dams which were fed corn oil exhibited a marked tendency to incorporate 22:6ω3 in the immediate postnatal period in spite of a relatively high linoleic-linolenic acid ratio in the milk.

INTRODUCTION

THE RELATIVELY HIGH CONCENTRATION of 4,7,10,13,16,19-docosahexaenoic acid (22:6ω3),[1] a metabolite of linolenic acid, is a characteristic of the brain lipids of many species (1,2,3). Moreover the level of this acid is maintained during nutritional studies in which little or no linolenic acid occurs in the diet (1,4). Weanling animals are employed in most nutritional experiments, and the brain lipids are formed before or immediately after birth and are relatively stable from a metabolic standpoint. The brain lipids must therefore reflect maternal lipids and hence maternal diet to a large degree. Since most stock-colonies are maintained on grain-type diets, linolenic acid metabolites would be expected to be present. The object of this study was to determine whether or not the docosahexaenoic acid is present in rats at birth or is synthesized during the immediate postnatal period, and to determine to what extent the level of this acid may be reduced in new-born rats by changing the maternal diet.

MATERIALS AND METHODS

Female rats of the Wistar strain were fed ad libitum from weaning to maturity either a stock grain diet or a semisynthetic diet containing 10% corn oil. The experimental animals consisted of young from the litters of these females.

Young rats were sacrificed at birth and approximately two and three weeks after birth. The brains were removed and individually homogenized in chloroform-methanol (2:1v/v) under an atmosphere of nitrogen. Extraction of the lipids was continued for 30 min, and the residue was re-extracted twice more with chloroform-methanol. The combined extracts were washed with 0.2 volumes of water to remove water-soluble components, the mixture was centrifuged, and the aqueous layer was removed. After drying over sodium sulfate, the extract was evaporated to dryness in vacuo. The lipid was dissolved in chloroform, filtered into a tared vial, the solvent was evaporated under a stream of nitrogen, and the weight of lipid was determined.

Methyl esters were prepared by refluxing the lipid with 14% boron trifluoride in methanol for 90 min. After cooling and addition of two volumes of water, the esters were extracted three times with petroleum-ether. Aldehyde dimethyl-acetals and sterol by-products were removed by thin-layer chromatography (5).

The esters were analyzed by gas-liquid chromatography on columns containing 15% ethylene glycol succinate-silicone copolymer (EGSS-X) on Chromosorb W (6). Peak areas were computed as the product of peak height and width at half-peak height. Results were expressed as weight percentage of total fatty acids.

The grain diet was extracted with ether for

[1] Abbreviation for polyunsaturated fatty acids, X:YωZ, where X, Y, and Z represent the number of carbon atoms, number of double bonds, and number of carbon atoms after the terminal double bond.

a period of 16 hr in a Soxhlet apparatus, the percentage of fat in the diet was determined, and the fatty acids were esterified and chromatographed as above. The fatty acid composition of the corn oil was also determined.

The fatty acid composition of the stomach contents immediately after suckling was determined as an indication of the fatty acid composition of the milk secreted by dams on the different diets. A chloroform-methanol extract of the stomach contents was employed, and esterification and chromatographic procedures were those described above.

RESULTS

The grain diet contained 3.0% of ether-extractable material. The major fatty acids in this diet (Table I) were linoleic, oleic, and palmitic, with lesser amounts of linolenic and stearic acids. Corn oil contained more linoleic and less linolenic acid than the grain lipids; however the total linolenic acid content of the corn oil diet was similar to that of the grain diet. More importantly from the standpoint of unsaturated fatty acid metabolism, the linoleic-linolenic acid ratios of the two diets differed considerably, being much higher in the corn oil diet (45:1 vs. 8:1).

Differences were noted in the fatty acid composition of the milk which was secreted by the dams (as determined by analysis of the stomach contents of the young). Milk from the grain-fed rats contained higher concentrations of the saturated acids; oleic and linoleic acids were higher in the milk from rats fed corn oil (Table I). The linolenic acid contents of the milk samples were similar, being only slightly higher on the grain diet and accounting for only about 1% of the total fatty acids. However the ratio of linoleic to linolenic was about three times higher on the corn oil diet than on the grain diet. A component with the retention characteristics of arachidonic acid was found in both samples but was higher in the corn oil group.

Table II indicates the effect of maternal diet and age on the total fatty acids of the brain in the young rat. In this experiment the animals were allowed to suckle their natural mothers. Analyses were carried out on rats at approximately 0, 12, and 24 days of age. Because of uncertainty in the actual time of birth, these ages may be in error by 24 to 36 hr.

In many respects the brain fatty acids of young rats from mothers on the two different diets were similar. Myristic, palmitic, and palmitoleic acids all decreased whereas stearic, oleic, and linolenic acids increased from birth to weaning. Changes in linoleic acid were small, increasing slightly on the grain diet, decreasing slightly on the corn oil diet.

TABLE I

Fatty Acid Composition[1] of Maternal Diets and Stomach Contents of Young

Acid	Maternal Diets		Stomach Contents	
	Grain	Corn oil	Grain	Corn oil
8:0			0.8	0.7
10:0			8.0	5.6
12:0			9.6	4.1
14:0	1.1		10.3	3.0
16:0	17.4	11.2	22.3	16.2
16:1	1.7	0.1	2.5	2.5
18:0	5.4	2.0	3.5	2.9
18:1	25.9	26.5	19.6	23.9
18:2	42.3	58.5	14.7	28.7
18:3	5.3	1.3	1.3	0.8
20:4 (?)			2.4	5.1
18:2/18:3	8.0	45.0	11.3	35.8

[1] Expressed as percentage by weight of total fatty acids. Minor components omitted from table.

The levels of arachidonic acid in the brains were similar on both diets and followed similar patterns, increasing slightly during the first 12 days, then decreasing again. Dietary differences were noted in the most highly unsaturated acids of both the ω6 and ω3 series. More 22:5ω6 was found at birth in the young rats from mothers fed corn oil. The level of this acid decreased during the first three weeks of life. On the grain diet this acid was initially lower and again decreased as the animals aged. At birth the major difference between young from dams on the two different diets was in the level of 22:6ω3 in the brain. It was considerably higher in the case of the grain diet. As the animals aged, the level of this acid increased on both diets. The increase was proportionately greater in the young from the dams which were fed corn oil but remained significantly lower in these animals at weaning.

In order to determine the extent to which maternal diet influenced the brain fatty acids of the young after birth, young animals were transferred at birth to foster mothers which were fed the second diet. The results (Table III) were quite similar to those in the initial experiment. Again the major differences and changes were associated with the polyunsaturated acids. Young born to dams fed corn oil and suckling grain-fed foster mothers had a high level of 22:5ω6 in the brain lipids at birth. The level of this acid dropped rapidly during the first three weeks of life, attaining a level similar to that in the young of dams

TABLE II

Brain Fatty Acid Composition[1] of Young Rats Suckling Natural Mothers

Time(days)	Grain diet			Corn oil diet		
Acid	0 (5)[2]	12 (3)	24 (3)	0 (4)	12 (3)	24 (4)
14:0	1.8 ± 0.05	1.1 ± 0.00	0.4 ± 0.00	2.0 ± 0.10	1.0 ± 0.03	0.4 ± 0.02
16:0	31.6 ± 0.62	29.4 ± 1.39	23.4 ± 0.48	32.6 ± 0.43	30.3 ± 0.43	24.1 ± 0.30
16:1	4.7 ± 0.26	2.8 ± 0.15	0.9 ± 0.06	4.9 ± 0.23	2.6 ± 0.03	0.8 ± 0.08
18:0	17.1 ± 0.42	18.5 ± 0.87	21.2 ± 0.35	17.1 ± 0.14	18.4 ± 0.10	21.2 ± 0.26
18:1	14.4 ± 0.26	15.1 ± 0.38	20.3 ± 0.12	16.1 ± 0.19	15.0 ± 0.02	20.3 ± 0.21
18:2ω6	0.8 ± 0.06	1.1 ± 0.08	1.2 ± 0.09	1.7 ± 0.24	1.2 ± 0.06	1.2 ± 0.02
18:3ω3	0.3 ± 0.03	0.3 ± 0.03	1.0 ± 0.12	0.3 ± 0.04	0.3 ± 0.03	0.9 ± 0.05
20:4ω6	13.1 ± 0.33	14.5 ± 0.17	12.7 ± 0.15	12.4 ± 0.31	16.2 ± 0.49	12.7 ± 0.60
22:4ω6	3.7 ± 0.16	3.5 ± 0.03	3.4 ± 0.09	3.4 ± 0.13	4.1 ± 0.12	4.4 ± 0.13
22:5ω6	2.1 ± 0.06	1.1 ± 0.07	0.6 ± 0.06	6.4 ± 0.26	5.7 ± 0.06	4.5 ± 0.23
22:6ω3	9.8 ± 0.18	12.0 ± 0.19	14.1 ± 0.23	2.8 ± 0.06	4.6 ± 0.03	8.7 ± 0.24

[1]Expressed as percentage by weight of total fatty acids ± S.E. Minor components omitted from table.
[2]Figures in parentheses indicate number of samples.

which were fed grain in the initial experiments (Table II). The former animals were also born with low levels of 22:6ω3 in the brain, but suckling grain-fed dams rapidly raised the level of this acid to that in the natural young from these dams. In contrast, rats born to dams which were fed grain but suckling those which were fed corn oil had low levels of 22:5ω6 in the brain, and this increased during the first three weeks of life to a level similar to that in the natural young from rats fed corn oil (Table II). The 22:6ω3 content of the brains of the young born to dams which were fed grain was relatively high at birth and decreased slightly as the animals suckled dams which were fed corn oil, achieving the level characteristic of the natural young from these dams (Table II).

One interesting feature of the second experiment was the relatively high and variable concentration of linoleic acid in the brain lipids at birth (Table III). The reason for this is not immediately apparent, but the possibility that it reflects the prenatal levels of this acid is being investigated.

DISCUSSION

It is apparent from Table II that the maternal diet exercised a considerable control over the level of 22:6ω3 in the brain lipids of new-born rats. When the maternal diet contained corn oil, with its high ratio of linoleic to linolenic acid, a relatively low level of this polyunsaturated acid was found. In contrast to the new-born of grain-fed dams, higher levels of 22:5ω6 occurred. These differences are compatible with the competitive nature of linoleic and linolenic acid metabolism proposed by Holman and Mohrhauer (7). The higher ratio of linoleic to linolenic acid in the corn oil diet would suppress elongation and desaturation of the latter to 22:6ω3 and favor formation of 22:5ω6 from linoleic acid. Inhibition of linolenic acid metabolism would be reduced by the grain diet with its lower linoleic-linolenic acid ratio.

TABLE III

Brain Fatty Acid Composition[1] of Young Rats Suckling Foster Mothers

Natural mother Foster mother	Corn oil diet Grain diet			Grain diet Corn oil diet		
Time(days) Acid	0 (3)[2]	10 (3)	23 (4)	0 (3)	10 (2)	23 (2)
14:0	2.2 ± 0.12	1.5 ± 0.07	0.3 ± 0.02	2.0 ± 0.22	1.1 ± 0.09	0.3 ± 0.06
16:0	28.4 ± 0.74	31.1 ± 0.90	21.4 ± 0.19	29.3 ± 0.96	28.3 ± 0.14	23.5 ± 0.79
16:1	4.0 ± 0.18	3.2 ± 0.07	1.0 ± 0.07	4.4 ± 0.26	2.8 ± 0.14	0.7 ± 0.04
18:0	14.1 ± 0.83	15.3 ± 0.45	21.0 ± 0.25	15.1 ± 0.71	17.6 ± 0.52	21.5 ± 0.49
18:1	18.8 ± 1.12	14.6 ± 0.35	19.6 ± 0.09	17.4 ± 0.18	14.8 ± 0.15	20.2 ± 0.37
18:2ω6	7.0 ± 2.51	2.3 ± 0.45	1.7 ± 0.45	3.7 ± 1.64	1.9 ± 0.24	1.2 ± 0.06
18:3ω3	0.4 ± 0.12	0.7 ± 0.20	1.1 ± 0.12	0.4 ± 0.00	0.5 ± 0.20	1.4 ± 0.02
20:4ω6	11.0 ± 0.35	14.3 ± 0.60	12.3 ± 0.04	11.4 ± 0.39	14.6 ± 0.24	12.7 ± 0.53
22:4ω6	3.4 ± 0.18	3.1 ± 0.16	3.7 ± 0.11	2.8 ± 0.24	4.2 ± 0.15	4.8 ± 0.21
22:5ω6	6.5 ± 0.48	2.2 ± 0.07	0.8 ± 0.05	2.4 ± 0.20	4.4 ± 0.39	5.3 ± 0.62
22:6ω3	1.8 ± 0.40	10.7 ± 0.10	14.7 ± 0.55	9.5 ± 0.21	8.0 ± 0.21	7.4 ± 0.89

[1]Expressed as percentage by weight of total fatty acids ± S.E. Minor components omitted from table.
[2]Figures in parentheses indicate number of samples.

During the first three weeks of life the level of 22:6ω3 increased in both dietary groups; however the increase was proportionately greater in the young from dams which were fed corn oil. This was somewhat surprising since the level of linolenic acid in milk was quite low (approximately 1% of total fatty acids). Moreover the linoleic-linolenic ratio was considerably higher in the milk from rats which were fed corn oil. The proportionately smaller increase in 22:6ω3 on the grain diet in spite of the lower linoleic-linolenic acid ratio in the milk could result from an absolute limitation on the amount of this acid which can occur in brain lipids. In this case the level found in these animals at 24 days would have approached this limiting value. The hexaenoic acid comprised 13.6% of the total fatty acids of adult rats which were maintained on a grain diet.

The relatively large increase in 22:6ω3 in the brains of the young from dams which were fed corn oil indicates highly preferential incorporation of this acid into the brain lipids during the first three weeks of life even though the linoleic-linolenic acid ratio of the milk was quite high. This ratio was lower in the milk than in the maternal diet however, and the rapid incorporation of 22:6ω3 into the brain during this period might be attributable to a reduced inhibition of linolenate metabolism by linoleate in comparison with the prenatal metabolism. The level of 22:6ω3 attained in 24 days was quite similar to the level of this acid in adult rats maintained on a corn oil diet (9.2%).

Alternate sources for ω3 acids incorporated into the brain lipids during the first three weeks of life are the other body tissues. It is possible that the 22:6ω3 present at birth in various body tissues could be accumulated in the brain during this initial growth phase. In this case the changes in the brain lipids would be independent of the milk fatty acids. Such was not the case in the present study. The final concentrations of the hexaenoic acid in rats which were suckling foster mothers (Table III) were similar to those occurring in the brain lipids of the natural young of the foster mother (Table II) in the initial experiment, thus indicating that the fatty acids received in the milk after birth influenced the composition of the brain lipids.

In the present study it was possible to control the level of 22:6ω3 in the brains of newborn rats by controlling the linoleic-linolenic acid ratio of the maternal diet. However there was a highly preferential incorporation of this acid into the brain lipids in the immediate postnatal period in spite of a highly unfavorable linoleic-linolenic acid ratio. In order to produce animals with low levels of this acid in the brain at weaning, it would be necessary to increase the linoleic-linolenic acid ratio of the maternal diet to a level considerably above that found in corn oil and possibly to eliminate linolenic acid from the diet completely.

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SHORT COMMUNICATIONS

Thin-Layer Chromatography of Plasma Lipids by Single Development

Many methods for the separation of the major classes of lipid by thin-layer chromatography are available, but all require double development with solvent mixtures of widely differing polarity. In the need to find a simple and relatively quick method for the separation of plasma lipids, the thin-layer method of Freeman and West (J. Lipid Res. 7, 324, 1966) has been modified with considerable advantage. Their long (34 cm) plates have been retained, but double development has been replaced by single development in a solvent system which completely separates into its individual components a mixture of phospholipid, monoglyceride, free fatty acid, 1:2 and 1:3 diglycerides, free cholesterol, triglyceride, and cholesterol ester.

All chromatographic solvents were distilled before use since their purification with activated alumina and their contact with filter paper was found to contaminate them and thus confound subsequent determination of the separated lipids by the dichromate-reducing method of Amenta (J. Lipid Res. 5, 270, 1964). The purity of individual lipid standards was checked by thin-layer chromatography.

Thin layers (0.4 mm) of Silica gel G (E. Merck A.G., Darmstadt, Germany) were prepared on 34x20x0.3-cm glass plates, and the absorbent was activated for 30 min at 100-105C before use. In practice several plates were prepared and activated at one time, then stored in a nondesiccated cabinet until required. A wide variation in the degree of activation was found to have negligible effect on the separation of lipid classes. Oxidizable impurities contained in the silica gel were removed by complete development of the blank plates with methanol prior to using them for the separation of plasma lipids. In this way the impurities were eluted beyond the solvent front of an analytical run and any interference in the determination of cholesterol esters was avoided.

The plates were run in a nonpresaturated S chamber set up in the following way. A strip of silica gel, 1 cm wide, was removed from the longest edges of two plates, and the remaining area of each plate was divided into three lanes each spaced 1 cm apart from the other. Standard mixtures and unknown samples of lipid were applied to the appropriate lanes, and the two plates were clamped, with spring paper clips, facing each other but separated by a third uncoated plate which was fitted with a length of longitudinally split silicone rubber tubing 4.8 mm ID and 1.6 mm in wall thickness (Griffin and George Ltd., Wembley,

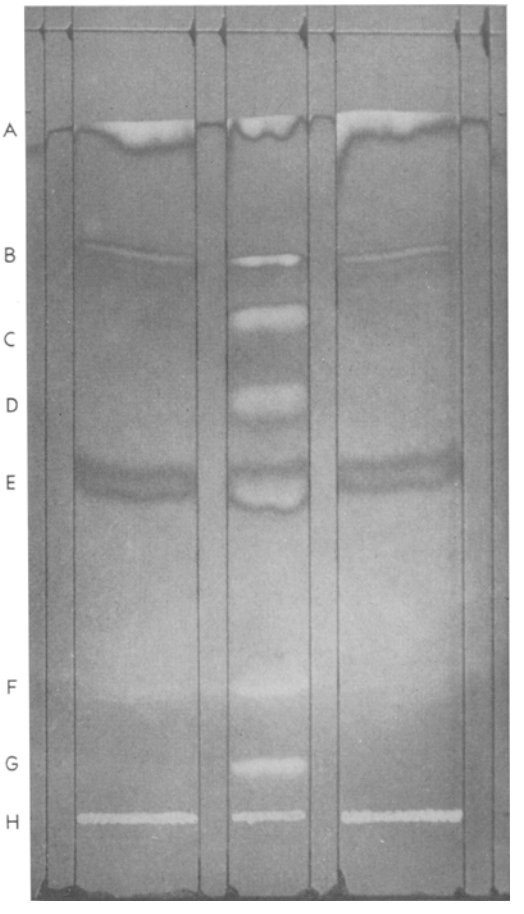

Fig. 1. Separation of 5 mg of a standard lipid mixture (center lane) and of 8 mg of lipids extracted from blood plasma of a lactating cow (outer lanes): A, cholesterol esters; B, triglycerides; C, 1:3 diglycerides; D, 1:2 diglycerides; E, free cholesterol; F, free fatty acids; G, monoglycerides; H, phospholipids. Developing solvent benzene, ether, ethyl acetate, and acetic acid in the proportions 80:10:10:0.2.

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Fig. 1 shows that the various lipids were clearly separated from each other; the Rf values obtained were phospholipid 0, monoglyceride 0.08, free fatty acid 0.20, free cholesterol 0.48, 1:2 diglyceride 0.59, 1:3 diglyceride 0.72, triglyceride 0.80, and cholesterol ester 1.00. The lipids were separated in sharp narrow bands, even when the maximum loading of 8 mg of total lipid was applied to the plate, thus facilitating their subsequent removal from the plate before quantitative determination. If the S chamber was used without the middle uncoated glass plate, the developing solvent did not run at the same speed on both plates, thereby giving slightly different separations.

Shorter runs of 15 cm on standard 20-cm plates by using the same chamber but the single solvent dichloromethane has also been found useful on a preparative scale for separating triglycerides and cholesterol esters from other classes of lipid. With this system the cholesterol esters run with the solvent front, triglycerides approximately at three-quarters of this distance, and the other classes remain either close to or at the origin. Separation is achieved in about 40 min.

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[Received April 25, 1967]

LETTER TO THE EDITOR

The Chain-Length Overlap Problem in Gas-Liquid Chromatography with Polyester Liquid Phases

Sir: Some confusion may have arisen with readers of this Journal through the inadvertent use in a recent communication of "arachidonate" in lieu of "arachidate," with the implication that 18:3ω3 could coincide with both 20:4ω6 and 22:0 (shorthand notation employed gives chain length, number of double bonds, and position of double bond farthest from carboxyl group relative to terminal methyl group). It was also implied that the separation of pairs of fatty acids coincident on a highly polar diethylene glycol succinate (DEGS) column could not be effected by temperature reduction and required a nonpolar (Apiezon) column for a second analysis (Nazir et al., Lipids *1*, 453, 1966).

These difficulties are part of a larger problem of "chain-length overlap" on polar liquid phases which, for convenience in discussion, may be restricted to even chain-lengths. To clarify the problem it should be noted that many lipid workers consider the separation of 18:0 from 18:1ω9 to be the determining factor in polyester polarity. Supina (in "Biomedical Applications of Gas Chromatography," ed. H. A. Szymanski, Plenum Press, New York, 1964, p. 271) divided polyester liquid phases into two groups on the basis of behavior on Gas-Chrom P coated at 15% by weight. The highly polar (Group 1) materials consisted of ethylene glycol succinate (EGS), DEGS, and a methyl organosilicone copolymer with succinic acid and ethylene glycol (EGSS-X). The 18:1ω9/18:0 separation factor for Group 1 was about 1.17. The medium polarity (Group 2) materials were ethylene glycol adipate (EGA), butanediol succinate (BDS), and possibly a methyl organosilicone copolymer with different proportions of succinic acid and ethylene glycol (EGSS-Y), as well as others. The

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TABLE I

Effect of Temperature and Percentage Polyester on Certain Separations in Gas-Liquid Chromatography of Methyl Esters of Fatty Acids

Liquid phase	DEGS[a,b]			EGSS-X[c]						
Percentage	20%			3%[d]		8%[e]				15%[e]
Temperature	150	170	190	170	160	170	180	190	205	175
Fatty acids						Equivalent chain-lengths				
18:3ω3	19.93	20.17	20.32	19.78	19.91	19.95	19.96	20.11	20.26	20.32
20:4ω6	21.68	21.96	22.33	21.58	.....	.....	.....	.....	.....	.....
22:1	22.05	22.24	22.41	22.08	.....	.....	.....	.....	.....	.....
						Separation factors				
20:0/18:0	2.18	1.98	1.75	2.04	2.06	2.00	1.95	1.84	1.77	1.77
20:1/18:1	2.06	1.90	1.75	1.95	.....	.....	.....	.....	.....	.....
18:1/18:0	1.15	1.16	1.15	1.12	1.14	1.14	1.14	1.15	1.15	1.17
20:1/20:0	1.09	1.12	1.15	1.08	.....	.....	.....	.....	.....	.....
22:1/22:0	1.03	1.09	1.13	1.03	.....	.....	.....	.....	.....	.....

[a] On Anakrom ABS.

[b] Ackman

[c] On Gas-Chrom P.

[d] Ackman and Burgher.

[e] Supina.

separation factor for 18:1ω9/18:0 was about 1.12 for these liquid phases. Supina's data clearly show that, with Group 1 liquid phases for these particular columns, 18:3ω3 falls after 20:0 whereas with the Group 2 phases it falls well before 20:0. The separation of 18:1ω9 and 18:0 is therefore related to the extent of chain-length overlap.

Although for equivalent columns and operating conditions Supina showed that the separation for two methylene units in saturated acid esters (e.g., 20:0/18:0) was slightly greater for Group 2 polyesters, it may be considered as an approximation that the separation of the normal saturated fatty acids will be the same for both high polarity and medium polarity phases. The extent of the chain-length overlap for commonly occurring fatty acids may then be illustrated by analyses of methyl esters of the fatty acids from a marine oil (cod liver oil, cf. Ackman and Burgher, JAOCS *42*, 38, 1965) in "expanded" and idealized form as shown in Figures 1 and 2.

In the high polarity (EGSS-X) analysis 20:4ω6 and 22:1 coincide, but 20:5ω3 may be determined readily since correction for superimposed C_{22} dienes and trienes, unless indicated by the medium polarity analysis, is not required. The coincidence of 18:3ω3 and 20:1 is also normal on high polarity columns. On the medium polarity (EGSS-Y) analysis, 20:4ω6 is free of conflicting C_{22} components but 22:1 and 20:5ω3 coincide. Internal normalization through a suitable internal standard, such as 16:0 or 18:1ω9, permits two separate calculations for 22:1. Usually these agree to 5% or better, or about the same accuracy as for an isolated peak of the same magnitude. In the medium polarity (EGSS-Y) analysis, 18:3ω3, 20:1, and 20:2ω6 may be determined without undue interference and this information may be used to determine 18:4ω3 and 20:0 from their coincident peaks in the high polarity (EGSS-X) analysis.

The polarity of Group 1 polyesters is usually quite dependent on the liquid phase percentage and support, but Group 2 polyesters may be less affected. Craig (in "Gas Chromatography, Proc. 3rd Int. Symp. Michigan State," ed. N. Brenner, J. E. Callen, and M. D. Weiss, Academic Press, New York, 1962, p. 37) explored this in detail and noted that, with BDS at all reasonable substrate/support proportions, 18:3ω3 preceded 20:0 on C-22 Firebrick, Celite 545, and Chromosorb W. By contrast EGS behaved the same way at less than 10% on Chromosorb W or 20% on C-22 Firebrick. At higher proportions of EGS, 18:3ω3 followed 20:0. Supina found that with 15% EGSS-X at 175C 18:3ω3 followed 20:0, with 8% EGSS-X the relative positions depended on temperature (Table I), and with a 1% EGSS-X column 18:3ω3 preceded 20:0. Ackman and Burgher found that on a 3% EGSS-X column at 170C 18:3ω3 also preceded 20:0.

Other groups have confirmed these findings with combinations of 20% EGS on C-22 Firebrick, 20% EGS on Chromosorb W (Grieco, Olearia *16*, 122, 1962) and 20% BDS on Chromosorb W (Buoncristiani et al., Olearia *16*, 99, 1962) and thus demonstrated the broad applicability of these classifications when due regard is paid to the three factors of liquid phase, support, and ratio of these two variables for conventional operating temperatures of 180-200C.

The aging of gas-liquid chromatographic columns inevitably results in losses of liquid phase and a reduction in chain-length overlap. As shown by Craig, this should produce, with Group 1 materials such as EGS, the equivalent of a medium polarity column, and this has been illustrated by Grieco for a column of 20% EGS on C-22 Firebrick, where the shift of 18:3ω3 from after to before 20:0 was accompanied by a change in the 18:1ω9/18.0 separation factor from 1.18-1.20 to 1.12-1.14. The aging of one DEGS column over a period of four weeks has been illustrated by Imaichi et al. (Bull. Soc. Exp. Biol. Med. *112*, 1085, 1963) and clearly shows the concurrent shortening of retention times for 18:3ω3 relative to 20:1 and of 20:4ω6 relative to 22:1. Since operating temperature and carrier gas pressure were constant while retention times in general decreased nearly 50%, it is evident that the initial 20% concentration of liquid phase (corresponding to a Group 1 polarity) must have fallen to the 10-15% concentration range (corresponding to a Group 2 polarity). Bleeding of liquid phases is usually more pronounced at the inlet end of the column and results in a concentration gradient along the column. Therefore conditioning and aging do not necessarily cause changes which can be related linearly to the percentage of liquid phase.

It was reported by Nazir et al. that a reduction in operating temperature with a DEGS column from 180 to 165C did not affect the coincidence of 20:4ω6 and 22:1. However a reduction in operating temperature of a similar column from 190 to 170C showed a small degree of separation (Table I). By further reducing the temperature to 150C, nearly complete separation of 20:4ω6 and 22:1ω9 was achieved (Ackman, J. Gas Chromatog. *1*, No. 6, 11, 1963). The reduction in temperature had thus reduced the effective polarity of the column slightly in terms of chain-length overlap among unsaturated fatty acids. Supina has also demonstrated a change in polarity with temperature for 18:3ω3 and 20:0 (Table I) with a column of 8% EGSS-X on 100/120 mesh Gas Chrom-P, and similar instances are discussed by Buoncristiani et al. It is important to note however that the separation of methyl esters of saturated fatty acids (e.g., 20:0/18:0) is greater at lower temperatures than is separation of the corresponding unsaturated fatty acids (Table I, illustrated by Ackman). Correspondingly the separation between 20:1 and 20:0, and between 22:1 and 22:0 becomes less as temperature decreases and there is risk that small amounts of 20:1 and 22:1 will be lost under the 20:0 and 22:0 peaks at low temperatures. This can be a handicap in

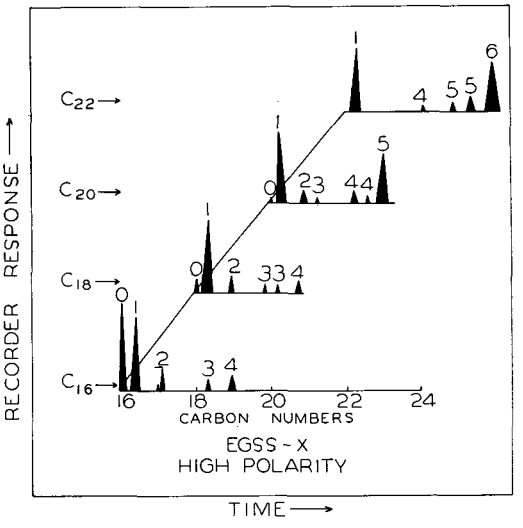

FIG. 1. Schematic gas chromatogram of methyl esters of fatty acids from cod liver oil. Analysis on 14% EGSS-X organosilicone polyester on 100-120 mesh Gas-Chrom P. Argon carrier gas at 14 psig, operating temperature 200C. Peak identifications give number of double bonds. With isomers the ω3 isomer follows the ω6 isomer. In the C_{18} acids ω values are 1, 4, and 7.

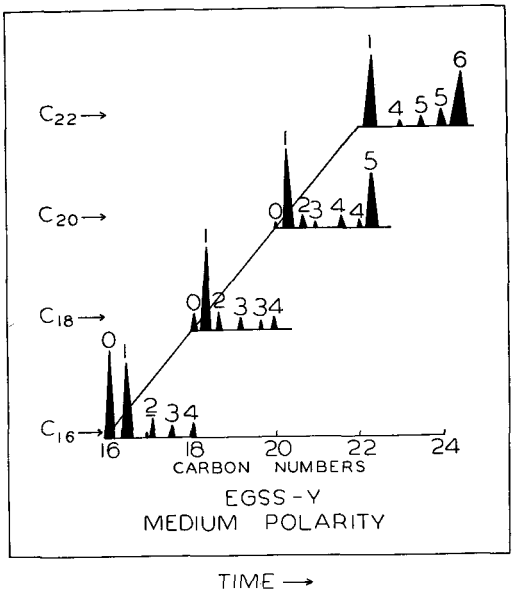

FIG. 2. Schematic gas chromatogram of methyl esters of fatty acids from cod liver oil. Analysis on 15.5% EGSS-Y organosilicone polyester on 100-120 mesh Gas-Chrom P. Argon carrier gas at 16 psig, operating temperature 200C.

analyses on polyester columns with a low percentage of liquid phase, which are usually operated at moderate temperatures in the range 160-180C (compare 20% DEGS at 150C and 3% EGSS-X at 170C).

Achievement of improved separation among pairs of unsaturated esters of different chain-lengths by altering operating temperature does not necessarily improve separations between unsaturated and saturated materials of either the same or different chain-lengths. Analysis of the same sample on a polyester column of a different degree of polarity is usually preferable since each analysis can be carried out at optimum column efficiency.

General principles governing retention times of aliphatic molecules of common chain-length dictate that, on all liquid phases, the reduction of ω values, for fatty acids of the same number of double bonds, usually increases retention time (e.g., 18:3ω6 and 18:3ω3 in Figs. 1 and 2). With polar liquid phases the addition of a methylene interrupted double bond normally increases retention time, and from the combination of the two effects there may be the coincidence of a few components of common chain-length and "normal" structures in analyses on polar liquid phases. Packed columns do not normally have enough efficiency to separate 20:3ω3 from 20:4ω6 although it may be calculated that 20:3ω3 (not shown in Figs. 1 and 2) would fall in the latter half of the 20:4ω6 peak (cf. Ackman, Ackman and Burgher, Ackman et al. Lipids *2*, 251, 1967). On nonpolar liquid phases, given the same ω value, an additional double bond usually decreases retention time. Thus, from the two effects, pairs of fatty acids C_x:nω3 and C_x:n-1ω6 will coincide on these columns, as shown by Nazir et al. The separation of common dienoic and trienoic acids from monoenoic acids may also be poor. In some cases, such as marine oils where dienoic C_{22} acids are usually insignificant components, the C_{22} monoethylenic acids may be determined. The saturated fatty acids are generally conveniently separated from other components, and accurate determinations are permitted.

The chief merits of nonpolar (Apiezon or SE-30) analyses are that there is no overlapping of the even chain-lengths. It is therefore possible to determine total chain-length composition without the necessity of hydrogenation (Smith and White, J. Lipid Res. *7*, 327, 1966).

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